Secondary Transmission Dynamics of the West Nile Virus in Mosquitoes

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Frontispiece/ Quote page

There has never been an era when mankind was without the nuisance of haematophagous insects and their blood sucking life cycle. These vampire-like pests and their disease spreading capabilities can be traced back to 2700BCE in ancient Chinese medical books. Malaria or a disease resembling malaria was described in the *Nei Ching*, edited by Emperor Huang Ti, more than 4,000 years ago (Centres for Disease Control and Prevention 2011). As the centuries passed numerous pathogens spread by the bite of an insect would become famous for their extremely high death toll. Epidemics of malaria, yellow fever, and the bubonic plague devastated populations without exposing the role of their insect transmitters. Bad air, the gods, rats, and water pollution were just some of the few things blamed for causing these terrible diseases. It wasn't until the general acceptance of the germ theory and the relentless work by scientists that the mysterious transmission was uncovered. The first discovery of insect pathogen transmission occurred in Hong Kong in 1878 when Sir Patrick Manson began feeding mosquitoes on his gardener who was suffering from a filarial disease (Centres for Disease Control and Prevention 2011). He was shocked when he observed the parasitic worm was actually flourishing within the dissected mosquito stomach. Perhaps the greatest result of Sir Manson's work was that it influenced Sir Ronald Ross' historic malaria discovery. Ross worked for several years without success trying to discover the malaria parasite within mosquitoes. In 1878 he had his breakthrough by determining that a specific *Anopheles* species was required to transmit the deadly disease. Just a few years later Dr. Carlos Juan Finlay (1881) conducted a study in Cuba in which he identified that yellow fever was transmitted by mosquitoes. Dr. Finlay was unaware of the work Sir Ross as his discovery was made through his own mental turmoil with the disease and its transmission

characteristics. Mosquitoes were not the only insect playing a role in epidemics around the globe at that time. An 1898 outbreak of the bubonic plague in Bombay, India tipped off Paul-Louis Simond that the local flea population had shifted from rats to human hosts when all the rats were dead from plague (Frith, 2012). Several of these men were ridiculed for the mere thought of their research. Yet they continued to pursue and test their hypotheses even when the initial attempts were unsuccessful. The following quotes are taken directly from letters, memoirs, and published works of the pioneers who founded vector borne research, a field that has since saved countless lives;

"I shall not easily forget the first mosquito I dissected. I tore off its abdomen and succeeded in expressing the blood the stomach contained. Placing this under the microscope, I was gratified to find that, so far from killing the Filaria, the digestive juices of the mosquito seemed to have stimulated it to fresh activity." **Sir Patrick Manson in an 1878 China Customs Medical Report**

Retrieved from (Venita, 2000).

"The belief is growing on me that the disease is communicated by the bite of the mosquito. …She always injects a small quantity of fluid with her bite- what if the parasites get into the system in this manner." **Sir Ronald Ross in an 1896 letter to Sir Patrick Manson.**

Retrieved from (Busvine, 2012).

"Lately, however, on abandoning the brindled and grey mosquitos and commencing similar work on a new, brown species, of which I have as yet obtained very few individuals, I succeeded in finding in two of them certain remarkable and suspicious cells containing pigment identical in appearance to that of the parasite of malaria. As these cells appear to me to be very worthy of attention … I think it would be advisable to place on record a brief description both of the cells and of the mosquitos." **Sir Ronald Ross' notes in the Dec 18th 1897 British Medical Journal.**

Retrieved from (Busvine, 2012).

"Yellow fever sometimes crosses the ocean and propagates in distant cities that boast quite different meteorological conditions from the center from which the infection stemmed; while, at other times, the same disease does not spread beyond a narrow epidemic area, irrespective of the fact that the meteorology and the topography of the surrounding areas show no differences that explain the distinct behavior of the same disease in two apparently similar sites. It was, therefore, impossible to look for that agent among microorganisms or zoophyte, because meteorological fluctuations that most affect the development of yellow fever exert little or no influence over these lowest categories of animate nature. To fulfill this first requisite, it was necessary to seek among insects and, bearing in mind that yellow fever is medically and also - according to recent works - histologically characterized by vascular lesions and physicochemical alterations of the blood, it seemed natural to look for an insect that could carry infectious particles from a sick man to a healthy man, among the ones who pierce blood vessels to suck human blood.

I finally asked myself, after considerations it is not necessary to mention here, if the mosquito could be the transmitter of yellow fever. This was the hypothesis behind the series of experimental studies I will expound." **Carlos Juan Finlay's 1881 speech to the Royal Academy of Medical, Physical and Natural Sciences in Havana, Cuba.**

Retrieved from (Finlay, 1881)

"On the rats captured alive, and on the rats which had just died, the fleas were thicker than I have ever seen them… We have to assume there must be an intermediary between a dead rat and a human. This intermediary might be the flea." **Paul-Louis Simond's observations of Bombay, India in 1897 for l'Institut Pasteur.** *Retrieved from (Frith, 2012)*

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Fiona F. Hunter. Her lectures sparked my interest in entomology as an undergraduate student and she encouraged me to further my education in the field I have become so passionate about. Fiona had faith in me beyond what my grade average showed, and for that I will be forever grateful. Aside from our professional relationship, Fiona has always made time for discussing any subject and always seemed able to make some sense of my many rants, I always felt better leaving a conversation with her.

Much of this thesis would not have been capable without the ample amount of help from Larissa Barelli. She was the go-to person when any trouble came up, she was able to find (or order) any missing equipment or reagents, and she has numerously been responsible for talking me off the proverbial ledge when things in the lab went astray.

I would also like to thank the core lab team of Bryan Giordano, Adam Jewiss-Gains, and Darrell Agbulos. I could not have imagined a better team of coworkers to experience this journey with and I am very grateful for the lifelong friendships that we have grown out of it. I'd also like to extend a thank you to all of the members of the Flylab who came and went during my time here, especially to Mariana Garrido de Castro as she dredged through the opening of the CL3 with us. I don't think the lab would be where it was today without her help.

Finally, to my friends and family thank you for being so understanding during this entire process. I know there are times when it seemed like I was more attached to the mosquitoes than I was to you, but your continued encouragement meant so much to me during the most stressful times.

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Glossary of Terms in relation to mosquitoes

Arbovirus: Viral pathogen transmitted through the blood feeding of an arthropod; technically, arthropod-borne virus.

Anautogeny: Female mosquito requires blood meal to produce eggs.

Autogeny: Female mosquito does not require blood meal to produce eggs.

Dead End Host: A vertebrate capable of becoming infected but not sufficient at amplifying the virus to a high enough titer for transmission to a subsequent mosquito feeding upon it.

Diapause: A phase where metabolic processes are reduced and development is stopped to allow the mosquito (egg, larva, or adult) an extended time to survive adverse conditions (e.g., winter).

Disseminate: Movement of a virus out of the midgut into different parts of a mosquito's body.

Emergence: The term used to describe the moment when a larvae hatches from an egg or when an adult mosquito breaks out of the pupae casing.

Epidemic: Spread of a disease that affects a large number of humans at the same time in a defined area.

Hematophagy: The process of ingesting and feeding upon blood.

Oviposition: The egg laying behaviour of female mosquitoes

Titer: Quantifiable concentration of virus within a defined sample. Generally expressed as plaque forming units per fluid volume (pfu/mL).

Mosquito Vector: Any mosquito species that has been shown to facilitate the survival (and often replication) of a pathogen within its body and is capable of transmitting it to a host.

Vector competence: Laboratory measure of the potential for a vector to become infected and subsequently to transmit a pathogen during a subsequent blood meal.

List of Abbreviations

DENV- Dengue Virus

ELISA- Enzyme-linked immunosorbent assay

ER- Endoplasmic reticulum membrane

JEV- Japanese Encephalitis Virus

KOUV- Koutango virus

KUNV- Kunjin virus

ISM- Infectious sugar meal

MIR- Minimum infection rate

MVEV- Murray Valley encephalitis virus

NY99 - The West Nile Virus lineage 1 strain identified from a New York patient

PFU- Plaque forming units

RT-qPCR- quantitative reverse transcriptase polymerase chain reaction

SLEV- St. Louis encephalitis virus

TOT- Transovarial or transovum transmission

VT- Venereal Transmission

WNV- West Nile Virus

WHO- World Health Organization

WNVL1- West Nile Virus lineage 1

WNVL2- West Nile Virus Lineage 2

YFV- Yellow Fever Virus

ZIKV- Zika virus

Abstract

West Nile Virus (WNV) is a member of the genus *Flavivirus* and is transmitted by mosquitoes. It first appeared in North America in New York City in 1999 and was first detected in Windsor Ontario in an infected bird collected in early August 2001. Prior to WNV, flaviviruses were not of major concern in Canada as only 1 short outbreak had occurred in the country. The secondary transmission cycles of WNV are typically overlooked in regards to their role in maintenance and survival of the virus in nature.

The first component of this thesis was to investigate transovarial or transovum transmission (TOT) of WNV in Ontario during the 2012 and 2013 epidemic seasons. 89 males from 7 different traps were tested from samples collected in 2012. During the 2013 season individuals across 9 different WNV vector species were collected throughout the Niagara region with 1023 tested for the presence of naturally occurring WNV TOT. None of the samples showed signs of TOT. A single gravid female *Culex pipiens* tested positive for WNV, but no viral RNA was found in any of her 270 offspring, highlighting the many difficulties in detecting this type of transmission in natural populations.

Laboratory testing was conducted to further investigate secondary transmission of WNV. A novel method for infecting adult mosquitoes was developed during this process; it was found more individuals would feed on an infectious sugar meal (ISM) as opposed to an infected blood meal in the lab setting. The ISM showed rates of infection, dissemination, and transmission by the WNV vector *Aedes albopictus* that were similar to those observed in previous vector competence studies. The ease of infecting adults and the similar rates of infection to previous literature suggests that this method could be used to investigate vector competence of other species for WNV.

The ISM was also successful in the infection of male *Aedes albopictus* and was used to investigate WNV venereal transmission (VT) within the species. Evidence showed that 45% of the uninfected virgin females were positive for WNV after mating with infected males, indicating for the first time that male *Aedes albopictus* could transmit the virus venereally.

Chapter One: Literature Review

Preface

The secondary transmission cycles of West Nile Virus (WNV) are typically overlooked in regards to their role in maintenance and survival of the virus in nature. This is mainly because male mosquitoes do not take a blood meal and it is assumed that there is a low rate of secondary transmission in nature. In recent years, however, there has been an increase in the literature that identifies flaviviruses surviving within naturally occurring secondary transmission cycles (Thongrungkiat et al. 2012; Rohani et al. 2007; Angel and Joshi 2008; Martins et al. 2012; Kenney and Brault 2014). This chapter will begin with a literature review on WNV transmission dynamics that are essential for understanding the methodology of the research for this thesis. The review will first provide a baseline understanding of the etiology, phylogenetics and life cycle of flaviviruses to highlight genetic and phenotypic similarities between WNV and other viruses in the genus *Flavivirus*. The subsequent sections will provide fundamental information on the mosquito and its life history traits, mainly focusing on biological processes involved in secondary transmission. The review will end with a critical analysis of several different methods used in laboratory studies involving WNV infection and detection within the mosquito vector. Based upon this review, the chapter will conclude by describing the overall objectives of this thesis.

1.1 Introduction to Vector Borne Disease

Vector borne diseases are illnesses caused by a diverse collection of microorganisms (bacteria, viruses, and protozoa) grouped by their parasitic nature and dual-host transmission cycle. The majority of these pathogens are transmitted through the bite of blood-sucking insects such as ticks, black flies, deer flies, sand flies, fleas, triatomine bugs, and mosquitoes (World Health Organization 2016). Pathogens transmitted by insects are ingested during blood feeding on an infected host and the pathogens may develop or reproduce within the vector before being transmitted to a new host during the next blood meal. The World Health Organisation (WHO) estimates that 17% of all infectious diseases are likely caused by one of the pathogens transmitted through the bite of an insect vector with approximately 1 billion clinical cases each year (World Health Organization 2016). Some of the notable diseases are dengue fever, malaria, human African trypanosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis, West Nile fever, and onchocerciasis (World Health Organization 2016). Combined these diseases caused by bacteria, viruses, and protozoa lead to the death of an estimated 1 million people globally each year (World Health Organization 2016). The term arbovirus (arthropod-borne virus) refers to the group of viruses transmitted through the bite of a blood feeding arthropod. Fitness of an arbovirus primarily hinges on its ability to transmit out of one host body and into the next before the host dies and its cellular environment no longer facilitates survival. The most common form of arbovirus transmission is by mosquitoes during the female blood meal. Immediately prior to feeding, an infected female mosquito expectorates saliva (containing virus) into the vertebrate host; during blood feeding, an uninfected female

can ingest virus-infected blood from an infected vertebrate host. This vector-host-vector transmission cycle is the primary transmission cycle of arboviruses and is considered epidemiologically significant for human infection. The largest group of arboviruses causing human disease come from the family Flaviviridae and includes over 40 species known to cause illness (Clements, 2011). West Nile Virus belongs to the family Flaviviridae and falls within the genus *Flavivirus.*

1.2 Family Flaviviridae Genus *Flavivirus*

Several viruses within the genus *Flavivirus* are quite similar in regards to their cellular processes, epidemiology, and genetic composition. The next section will highlight these similarities and investigate the viruses at the genetic and cellular levels.

1.2.1 Distribution

The name *Flavivirus* is derived from the Latin word *flavus* which means ‘yellow’. The name was given to the genus because of the historically famous Yellow Fever Virus in which patients expressed a distinct jaundice colouration (Mukhopadhyay et al. 2005). It is the largest and most well-known of the three Flaviviridae genera with approximately 70 distinct viruses in it. At least 40 of these are known to cause human disease and complications such as undifferentiated fever, capillary leakage (hemorrhagic disease), and neurological or encephalitis disorders (Daep et al. 2014; Diamond, 2009). There are several closely related disease-causing viruses within the genus: Dengue Virus (DENV), Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV), St. Louis encephalitis (SLEV), West Nile Virus (WNV), and Zika virus (ZIKV) to name a few. Bhatt et al.

(2013) suggest that annually there are between 67-136 million clinical cases of dengue fever and over 3.9 billion people are at risk of infection in over 121 countries (Brady et al. 2012). Rios et al. (2012) provide a global distribution map (Figure 1.1) for several medically important flaviviruses.

Until 1999 flaviviruses were generally not of a major health concern in Canada. There have only been two known flaviviruses to cause epidemics on Canadian soil since the development of the yellow fever vaccine in the 1930s (Calisher, 1994). The first was an epidemic of SLEV that occurred between 1975 and 1976 but only a few sporadic cases have been identified since (Calisher, 1994; Mahdy et al. 1979). In 1999 an epidemic of WNV broke out in New York City and quickly spread across North America and into Canada by 2001.

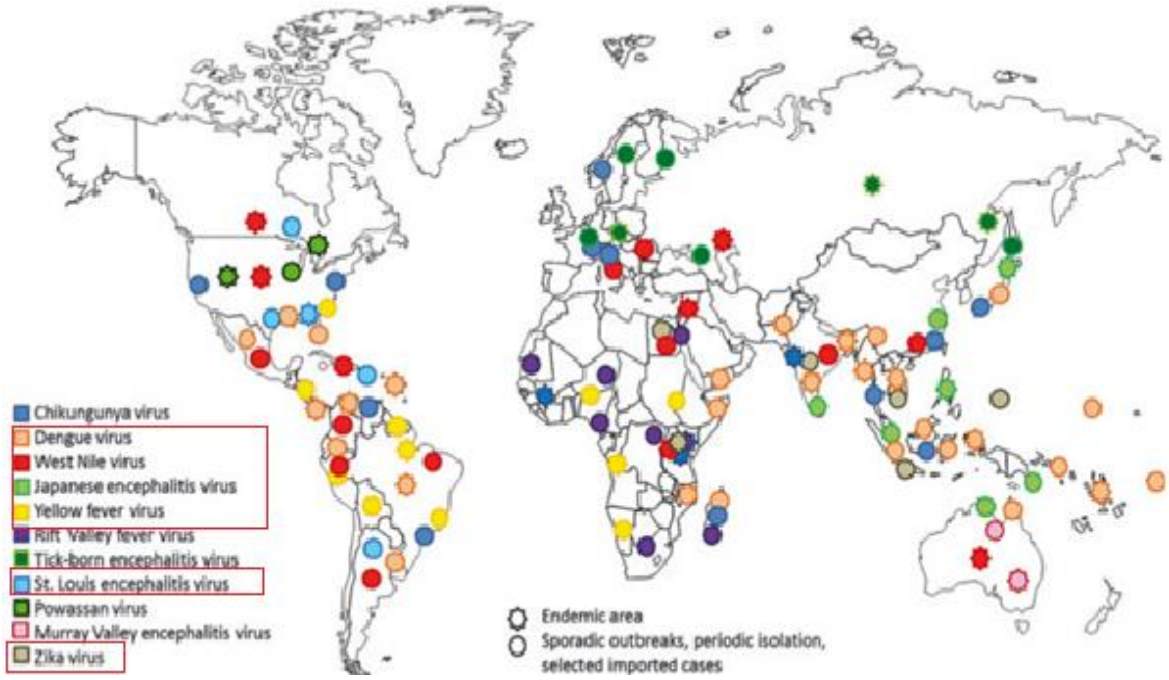

Figure 1.1- Global distribution of emerging and re-emerging arboviruses as of 2012. Viruses within the red boxes are from the genus *Flavivirus* (Modified from "Dengue virus and other arboviruses: A global view of risks" by Rios et al., 2012, ISBT Science Series, 7(1), p275. Copyright 2012 by the International Society of Blood Transfusion.)

1.2.2 Phylogenetic Relationships

Classifying the largest genus of the Flaviviridae family has been quite a difficult process; several approaches have been used, separately and in combination, to divide the genus into groups. The taxonomic relationships of these groups are constantly assessed and restructured to reflect advances in analytical methods and the inclusion of newly discovered viruses in the family (Moureau et al. 2015). A review of *Flavivirus* phylogeny by Kuno et al. (1998) describes the difficulty in classifying viruses within genus *Flavivirus* including factors such as the large number of distinct viruses, their wide geographic distribution, the diversity of arthropod vectors and vertebrate hosts, as well as how closely related several of them are antigenically (which can lead to serological cross reactions).

The most widely accepted phylogenetic tree of the genus *Flavivirus* (Figure 1.2) is commonly divided into four major clades based on the vectors known to transmit them; these are the mosquito-borne (MBFV), tick-borne (TBFV), insect-specific (ISFV), and no-known-vector (NKV) clades (Kuno et al. 1998; Gaunt et al. 2001; Moureau et al. 2015). The MBFV clade is the largest of the four with over 20 viral species, many of which are known for the human diseases they cause (Huhtamo et al. 2014). The clade is further subdivided into two groups based on a combination of their primary vector, vertebrate reservoir, and associated human disease. The first group are viruses mostly transmitted by *Culex* mosquitoes (*Culex*-spp. clade), known for having birds as their primary reservoir and for causing encephalitic diseases in humans (Gaunt et al. 2001; Huhtamo et al. 2014; Moureau et al. 2015). The second group is mostly isolated from *Aedes* mosquitoes (*Aedes*-spp. clade) with primates as their primary reservoir; they are

associated with hemorrhagic diseases in humans (Gaunt et al. 2001; Huhtamo et al. 2014; Moureau et al. 2015).

Another approach divides the genus based on diagnostic identification of antibodies in the serum forming nine different serocomplexes or serogroups. Five of these serogroups are composed strictly of MBFV designated as Dengue, Uganda S, Ntaya, Yellow Fever, and Japanese Encephalitis (Poidinger et al. 1996). Of these, Japanese encephalitis is the largest serogroup and includes 11 viruses (9 distinctly different viruses with 2 additional strains or subtypes); these are WNV and its subtype Kunjin virus (KUNV), SLEV, JEV, Murray Valley encephalitis virus (MVEV) and its subtype Alfuy virus (ALFV), Cacipacore virus (CPCV), Koutango virus (KOUV), Usutu virus (USUV), Kokobera virus (KOKV), and Stratford virus (STRV) (Poidinger et al. 1996; Mackenzie et al. 2006). All of these viruses fall within the MBFV *Culex*-spp. clade but are additionally grouped together in the Japanese encephalitis serocomplex because of the similarities they show in cross-neutralization tests using polyclonal sera (Poidinger et al. 1996).

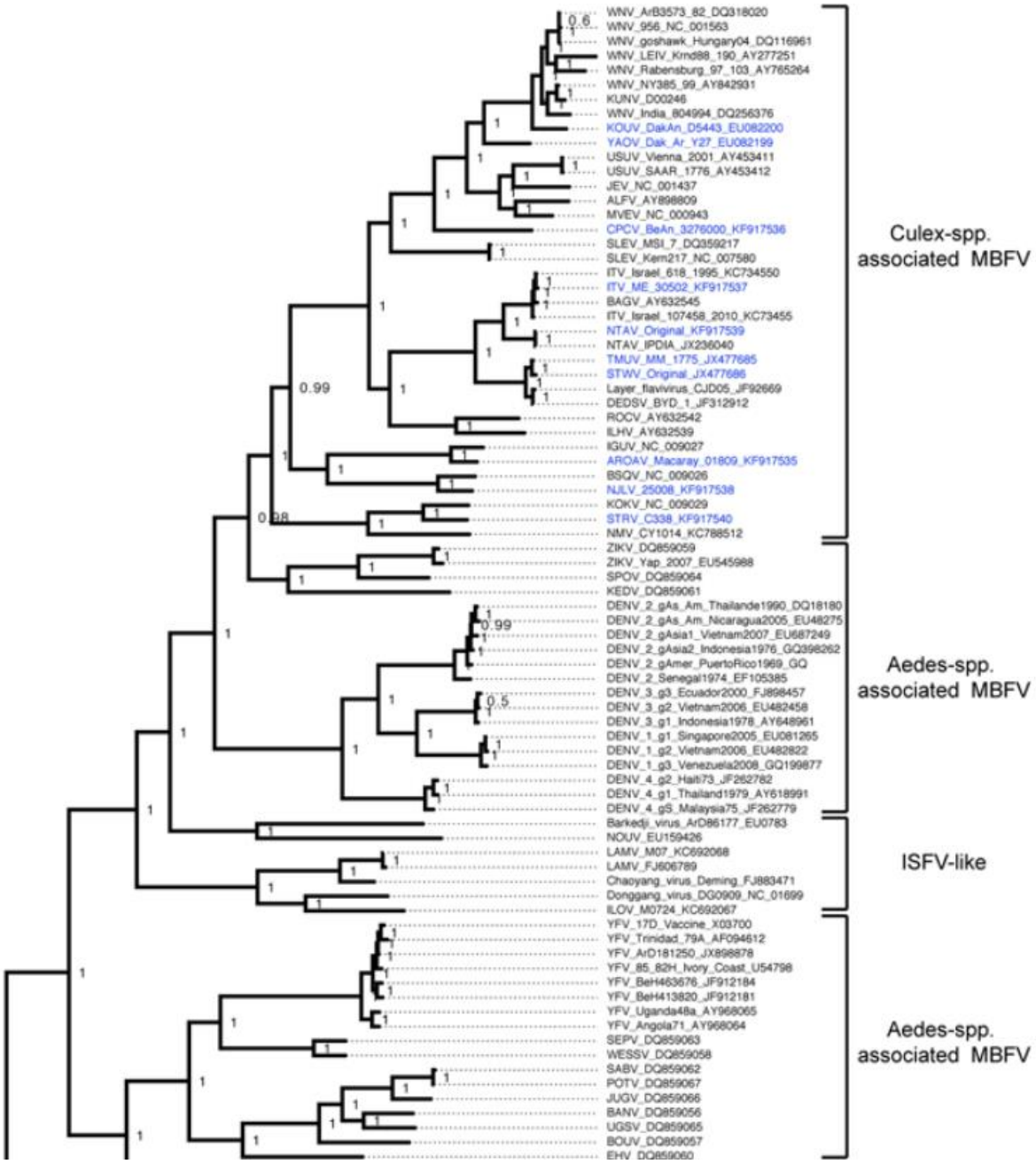

Figure 1.2- *Flavivirus* phylogenetic tree created using the complete genome from the E to NS5 region. The sequences compared were either derived by the authors or from those already found in Genbank. (Retrieved from "New Insights into Flavivirus Evolution, Taxonomy and Biogeographic History, Extended by Analysis of Canonical and Alternative Coding Sequences" by Moureau et al., 2015, PLoS ONE, 10(2), p5.

1.2.3 Genome

Structurally, flaviviruses are composed of a single 40-60nm spherical particle known as a virion (Perera et al. 2008). The *Flavivirus* genome is a single molecule of linear single-stranded RNA with approximately 10-12kbps (Suthar et al. 2013). It contains an open reading frame with a total of ten genes flanked by 5' and 3' terminal non-coding regions (Perera et al. 2008; Suthar et al. 2013). Three of these genes code for the structural components of the virion itself and the other seven code for non-structural functions of the virus. A schematic drawing of the *Flavivirus* genome (Figure 1.3) by De Filette et al. (2012) aids in understanding the exact placement of the *Flavivirus* genes. Closest to the 5' end are the three structural genes that encode the physical components of the virion: the protein capsid, immature precursor protein that is cleaved to form the mature membrane, and the envelope protein. Directly following these are the seven non-structural protein genes - NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 - which code for functions such as genome translation, genome replication, virion construction, different viremic interactions and nucleotide folding (Perera et al. 2008; Rios et al. 2012; Suthar et al. 2013; and Daep et al. 2014)

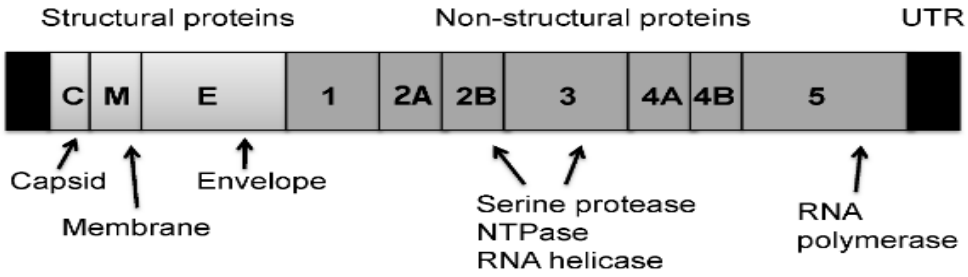

Figure 1.3- A schematic drawing of the *Flavivirus* genome and the arrangement of its genes (Retrieved from "Recent progress in West Nile virus diagnosis and vaccination" by De Filette et al., 2012, Copyright 2012; licensee BioMed Central Ltd.)

1.2.4 Virion

The virions of all flaviviruses have the same structural components as well as the same method of replication within their host cells. The genome of the virus is packaged into the protein capsid to form the nucleocapsid (~25-30nm) at the core of the virion (Suthar et al. 2013). Two structures surround the nucleocapsid core, namely, the membrane protein and the envelope. These structures have a built-in mechanism altering their formation as the virion exits the cell and enters the intercellular space (Mukhopadhyay et al. 2005). The first phase occurs within the cell directly following the assortment of the virion particle, otherwise known as an immature virion (Mukhopadhyay et al. 2005). Immature virions are characterised by the precursor membrane that surrounds the nucleocapsid. Precursor cleavage is activated by furin before exiting the cell, thus producing a mature virion as the nucleocapsid now becomes surrounded by an inner protein membrane and an outer envelope membrane (~15-30nm) (Mukhopadhyay et al. 2005).

1.2.5 Host-mediated Cellular Reproduction

The survival of a flavivirus is a dynamic process in which the virus must infect a host cell in order to replicate itself into numerous progeny virions that will then repeat the process in new cells. Although this is not fully understood, Perera et al. (2008), Daep et al. (2014), and Rios et al. (2012) agree that a virion attaches and enters a host cell through receptor mediated endocytosis (Figure 1.4- A). A drop in pH initiates the disassembly of the virion and its fusion to the endosome vacuole (Figure 1.4- B). This will break open the nucleocapsid and release the viral genome into the cytoplasm of the cell. At this point

the viral RNA can undergo one of two different processes. It is either replicated into a negative-sense RNA that can be copied to make numerous positive-sense RNA strands (Figure 1.4- D) or it is translated into a single long polyprotein on the endoplasmic reticulum (ER) membrane (Figure 1.4- C). This large polyprotein is cleaved into smaller structural and non-structural components necessary to complete assembly and maturation of a virion. The newly produced parts and replicated genome are packaged into the capsid proteins and enveloped in the ER producing an immature virion (Figure 1.4- E). Immature virions are transported through the low pH, trans-Golgi network where the envelope is glycosylated and the precursor membrane is cleaved to produce mature virions (Figure 1.4- F). The mature virions exit the cell through exocytosis and can now attach to another host cell to repeat the process (Figure 1.4- G).

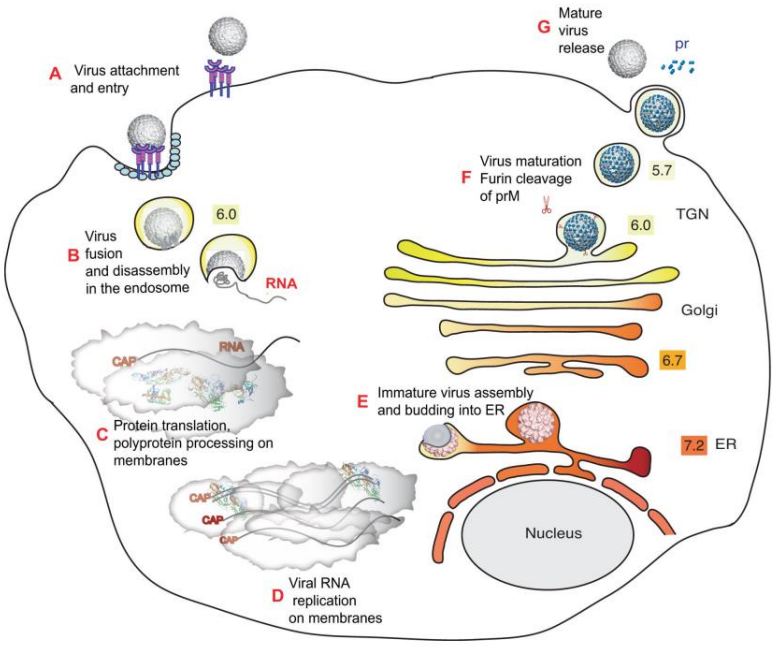

Figure 1.4- *Flavivirus* Life cycle. Numbers shown in coloured boxes refer to the pH of the respective compartments. (Retrieved with permission from "Closing the door on flaviviruses: Entry as a target for antiviral drug design" by Perera et al., 2008, in Antiviral Research, 80, p13.)

1.2.6 Extrinsic Incubation

Within the cell, the viral replication and amplification process (Section 1.2.5) requires sufficient time to complete. Given the ideal cellular environment and enough time a single virion can replicate exponentially. The time it takes for virus to infect and become disseminated throughout the mosquito is known as the extrinsic incubation period (Anderson et al. 2010). Anderson et al. (2010) identified a high correlation between the time it takes for infection and dissemination depending on the initial concentration of virus ingested, ambient temperature, and time since the initial infection. They suggest at least 10 days are required as a sufficient extrinsic incubation period when conducting oral infection with WNV on most vector species.

1.3 West Nile Virus

WNV has emerged as the most important *Flavivirus* to threaten the Canadian public. First, several traits of the historical WNV epidemics will be discussed and explained by investigating the genetics of the virus. This will be followed by a detailed look at the virus' primary transmission cycle, protocols used to identify the virus within the vector, and the species of mosquito known to transmit it.

1.3.1 Epidemic History

From the time of its discovery in 1937 up to the late 1990's WNV was considered an unimportant human pathogen (Gubler, 2007). The first epidemics after its discovery were sporadic as it spread throughout Africa, the Middle East, Europe and Asia (Gubler, 2007). Initially outbreaks were hard to identify as patients rarely showed severe West

Nile symptoms with most being either asymptomatic (showing no symptoms) or exhibiting only mild febrile illnesses (with patients rarely seeking medical help) (Gubler, 2007). This non-viremic perception changed in the mid 1990's as WNV emerged as a major global threat with severe cases of West Nile fever become increasingly frequent accompanied by a large increase in the number of patients suffering from neurological encephalitis (Gubler 2007). Petersen and Roehrig (2001) identified three alarming trends in WNV epidemiology during the late 1990's: The first was an increase in the frequency of human and equine outbreaks recorded from Romania and Morocco in 1996, Tunisia in 1997, Italy in 1998, and Russia, the United States, Israel, and France in 1999; the second was that the frequent outbreaks had a drastic increase in the number of severe human cases recorded; and the third trend was that human outbreaks of the virus were associated with an alarming increase in avian death rates in both New York State and Israel. Since its arrival in Canada during the 2001 season there have been over 5,000 reported clinical cases (Public Health Agency of Canada, 2016). Migratory flight patterns of avian hosts explain the large dispersal of WNV across continents in such short periods of time (Kramer et al. 2008).

Shortly following the 1999 New York City outbreak, Health Canada created the WNV National Steering Committee in order to establish guidelines for country wide surveillance and response so that a coordinated system was in place to monitor for WNV and respond if it did, in fact, travel into Canada (Drebot et al. 2003). The WNV mosquito surveillance program was established and running by the start of the 2000 field season and by 2002 the program documented WNV in Ontario, Quebec, Manitoba, Saskatchewan, and Nova Scotia. By the end of 2002, WNV had been detected across 44

U.S. states and 5 Canadian provinces making this encephalitis outbreak the largest arbovirus epidemic in North America and the world's largest WNV outbreak ever recorded (Drebot et al. 2003).

1.3.2 Phylogenetics

Phylogenetic analysis of viruses isolated from patient blood has identified two lineages of WNV. A more viremic strain evolved from the original WNV Uganda isolate and was responsible for the increase in epidemics that occurred in the 1990's (Figure 1.5). Ciota and Kramer (2013) suggest that the two lineages have about a 20% divergence between them with lineage 2 (WNV L2) being less neuroinvasive than lineage 1 (WNV L1). WNV L2 dispersed southward out of Uganda remaining mostly within the African continent, whereas WNV L1 initially spread northward causing epidemics in and around Europe before diverging into a second strain that caused epidemics in the Middle East and eventually spread into North America (Lanciotti et al. 1999; Ciota and Kramer, 2013).

The initial virus strain detected in North America was named NY99 after its discovery during the 1999 New York City outbreak, but this is for taxonomic purposes only as the NY99 strain has been found over a broad geographic range (Drebot et al. 2003; Ciota and Kramer, 2013). Soon after the isolation of NY99, Hindiyeh et al. (2001) compared it to virus isolates from patients around the world and determined that NY99 was genetically most related to one of the two WNV L1 strains circulating in Israel during their 1999 outbreak. These two outbreaks were both associated with an abnormally large death toll among bird populations and a high occurrence of neurological

disease indicating the newly diverged WNV epidemic strain was highly associated with increased viremia (Hindiyeh et al. 2001; Petersen et al. 2001; Ciota and Kramer, 2013).

There is a growing amount of evidence that shows the initial NY99 strain that arrived in North America has since evolved into a more virulent strain of the virus. Davis et al. (2005) compared similarities between 74 envelope protein isolates and 25 complete genome isolates collected across North America between 2001 and 2004. Their phylogenetic analysis showed a high relatedness between two groups of isolates: those collected prior to and those collected after 2002. Davis et al. (2005) suggest that a dominant strain of WNV emerged in North America prior to the 2002 season pointing to evidence of fixed nucleotide mutations among the latter group. It is important to note that the first major North American WNV epidemic following its introduction in 1999 was during the 2002 season. Phylogenetic analysis of North American virus collections from 1999-2005 led to the classification that a dominant variant (WNV 2002) had emerged across much of the continent (Davis et al. 2005). WNV 2002 is considered to be characterised by higher viremia epidemics compared to the initial NY99 strain (Davis et al. 2005).

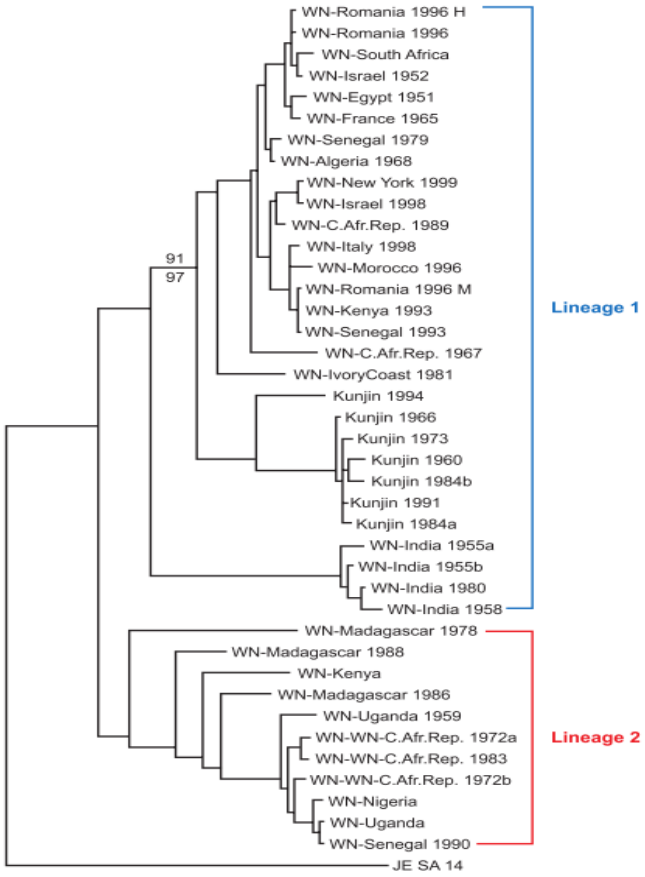

Figure 1.5- WNV phylogenetic tree based off of differences in the envelope glycoprotein. (Retrieved with permission from "Origin of the West Nile Virus Responsible for an Outbreak of Encephalitis in the Northeastern United States" by Lanciotti et al., 1999, Science, 286 (5448), p2333-2337. Copyright 1999, The American Association for the Advancement of Science).

1.3.3 Transmission Cycle

Typically, flaviviruses exist in a dual-host cycle between the arthropod vector and some vertebrate host such as a bird, rodent, amphibian, or primate (Kramer et al. 2008; Kenny and Brault, 2014). Infection through a blood meal is dose-dependent and thus only vertebrate hosts that amplify the virus to sufficient titers can contribute to the transmission cycle (Kenny and Brault, 2014). WNV primarily exists in an enzootic amplification cycle between mosquitoes and birds (Figure 1.6). Humans or equines

infected with WNV are not capable of amplifying the virus to a high enough titer so that it can be transmitted to another mosquito; these species are therefore incidental or dead-end hosts in the transmission of the virus (Gubler, 2007). Over 100 species of birds have been identified as WNV reservoirs (Kramer et al. 2008). Komar et al. (2003) exposed 25 North American species of birds to WNV and determined that the five most competent species were from Order Passeriformes: Blue Jay (*Cyanocitta cristata*), Common Grackle (*Quiscalus quiscula*), House Finch (*Carpodacus mexicanus*), American Crow (*Corvus brachyrhynchos*), and House Sparrow (*Passer domesticus*).

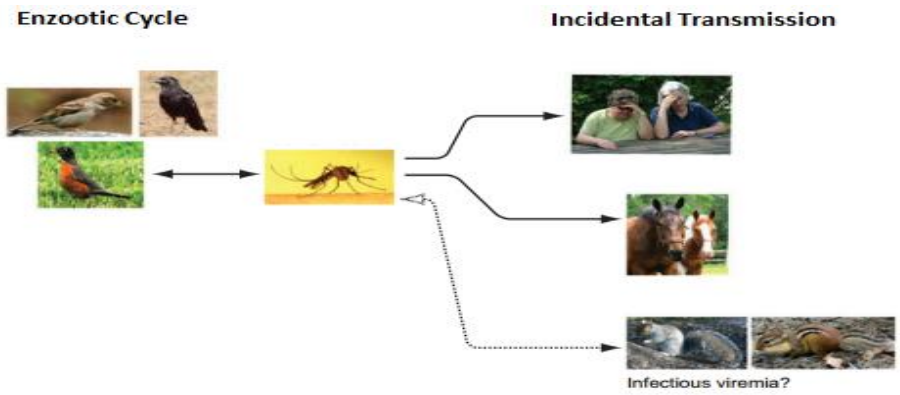

Figure 1.6- Primary transmission cycle and incidental vertebrate hosts of WNV (Retrieved with permission from "A Global Perspective on the Epidemiology of West Nile Virus" by Kramer et al., 2008, in Annual Review of Entomology, 53, p67. Copyright 2008, Annual Reviews)

In North America alone the virus has been detected in at least 65 different mosquito species (Appendix 1) (Kramer et al. 2008; CDC, 2012). The primary mosquito vector in temperate countries is *Culex pipiens* (Gubler, 2007). Kramer et al. (2008) suggest this distinction has been given to *Cx. pipiens* because it feeds almost solely on birds during the early season with a shift to a wider variety of hosts in late summer and early fall. This shift was correlated with epidemiological data in a field study by

Kilpatrick et al. (2006) providing evidence that an increase in human cases of WNV was linked to an increase in the number of *Cx. pipiens* feeding on humans.

1.3.4 Vector Species

A mosquito species' susceptibility to infection and ability to transmit a virus into a new host is considered its vector competency (Kenney and Brault 2014). Vector competence studies are used to identify and help compare the potential impact different species play in the natural transmission of a virus. Although *Cx. pipiens* is suggested to be the main WNV vector in North America, researchers have identified the virus in natural populations of at least 64 other species (CDC, 2012) with several identified to be transmitting the virus in North America (Table 1.1) (Turell et al. 2005). Of particular interest in Table 1 are two Asian mosquitoes that have become invasive in North America, namely, *Ochlerotatus japonicus* and *Aedes albopictus.*

Oc. japonicus is native to Japan, Taiwan, China, and Korea, and was first detected in New York State in 1998 (Andreadis and Wolfe, 2010) and Canada in 2001 (Thielman and Hunter, 2006); subsequently it has become well established and is now among the most abundant species collected in natural populations in Ontario (Bryan Giordano, personal communication). Several studies suggest that its strong establishment in Canada and rapid range expansion is due to its ability to produce freeze- and desiccation-tolerant eggs, its use of artificial containers as a main source for oviposition, and its aggressive behaviour at the larval stage thereby out-competing local mosquitoes for available resources (Thielman and Hunter, 2006; Andreadis and Wolfe, 2010).

Table 1.1- Potential for selected North American Mosquitoes to transmit WNV based on bionomics, vector competence, and virus isolations (Modified with permission from "An Update on the Potential of North American Mosquitoes (Diptera: Culicidae) to Transmit West Nile Virus" by Turell et al., 2005, in Journal of Medical Entomology, 42(1), p60. Copyright 2005, Entomological Society of America)

Species	Host preference	Activity time	Flight range	Vector competence for WNV	Field isolations of WNV	Potential to serve as a	
						Enzootic vector	Bridge vector
Ae. aegypti	Mammals	Crepuscular/day	200 m	+++	+	0	+
Ae. albopictus	Opportunistic	Crepuscular/day	200 m	++++	+	+	++++
Ae. vexans	Mammals	Crepuscular/night	>25 km	++	+++	0	++
Cq. perturbans	Opportunistic	Crepuscular/night	5 km	+	+	+	+
Cs. melanura	Birds	Crepuscular/night	9 km	+	++	++	0
Cs. inornata	Mammals	Crepuscular/night	2 km	+++	+	+	++
Cx. stigmatosoma	Birds	Night	1 km	+++	0	+++	+
Cx. erythrothorax	Opportunistic	Crepuscular/day	<2 km	++++	0	++	+++
Cx. nigripalpus	Opportunistic[f]	Crepuscular	5 km	++	+++	+++	++
Cx. pipiens	Birds	Crepuscular/night	2 km	+++	++++	+++++	++
Cx quinquefasciatus	Birds	Crepuscular/night	2 km	+++	0	++++	++
Cx. restuans	Birds	Crepuscular/night	2 km	++++	+++	+++++	++
Cx. salinarius	Opportunistic	Crepuscular/night	10 km	++++	+++	+++	++++
Cx. tarsalis	Opportunistic[f]	Crepuscular/night	>6 km	++++	++++	++++	+++
Oc. atropalpus	Mammals	Day and night	1 km	++++	+	+	++
Oc. canadensis	Mammals	Day	2 km	++	+	0	++
Oc. cantator	Mammals	Day	>10 km	++	+	0	++
Oc. dorsalis	Mammals	Day and night	5 km	+++	+	0	++
Oc. japonicus	Mammals	Crepuscular/day	unk	++++	+++	+	++++
Oc. melanimon	Mammals	Day and night	>10 km	+++	0	0	++
Oc. sierrensis	Mammals	Crepuscular/day	1 km	+	0	0	+
Oc. sollicitans	Mammals	Crepuscular/night	>25 km	++	+	0	+
Oc. taeniorhynchus	Mammals	Day and night	>25 km	+	+	0	+
Oc. triseriatus	Mammals	Day	200 m	+++	++	0	+++
Ps. ferox	Mammals	Day	2 km		+	0	0

Ae. albopictus is another invasive mosquito from Asia that has become well established on all temperate and tropical continents (Paupy et al. 2009). After arriving on the east coast of the United States, *Ae. albopictus* has become established in several of the states boarding Ontario, including New York, Pennsylvania, and Ohio (Ogden et al. 2014). Public Health Ontario's mosquito surveillance program identified 4 adult *Ae. albopictus* in between the 2005 and 2012 field seasons (PHO, 2015). Experts maintain the stance that the species is not established in Ontario and that these few adults were most likely caught in a northern wind current blowing them into the province or from

unhatched eggs surviving in shipped materials (Ogden et al. 2014). Surveillance collections from the 2016 season found *Ae. albopictus* in western Ontario surviving at all three life stages within field samples. Again experts believe that the individuals collected were from an isolated population (Bryan Giordano, personal communication), but there is potential for the egg stage to survive through the winter. A plethora of distribution information on its northward spread in Europe and Asia is linked to an increase in northern climate change (Caminade et al. 2012; Kobayashi et al. 2002; Paupy et al. 2009; Ogden et al. 2014). Similar to *Oc. japonicus,* the eggs of *Ae. albopictus* are tolerant to freezing and desiccation, the eggs can remain in diapause for long periods, the larval stages are excellent competitors with other species for resources, and the species has rapidly adapted to urban environments by utilizing both natural and artificial containers for oviposition (Sota and Mogi 1992).

Ae. albopictus is considered a rural and suburban mosquito that feeds opportunistically on a wide variety of vertebrates in nature (Richards et al. 2006; Paupy et al. 2009). Their feeding behaviour is considered aggressive and occurs mainly during daylight hours making them of high risk to propagate zoonotic pathogens between animals and humans (Richards et al. 2006; Paupy et al. 2009). *Ae. albopictus* has been identified as a competent vector of over 20 arboviruses (Gratz 2004) and considered an excellent vector of several flaviviruses such as SLEV, JEV, DENV, YFV and WNV (Paupy et al. 2009). In North America, wild populations have tested positive for the presence of WNV in Pennsylvania, New Jersey, and the D.C Maryland area (Gratz 2004). Laboratory studies have shown *Ae. albopictus* is susceptible to oral infection from avian hosts (Turell et al. 2001) and through membrane feeding (Akhter et al. 1982). They have

also been shown in laboratory to transmit WNV into uninfected avian hosts 14 days post infection (Turell et al. 2001).

1.4 Mosquito Biology

Mosquitoes are classified as true flies belonging to the order Diptera, family Culicidae. Several aspects of a mosquito's life cycle influence the ability of WNV to be transmitted through these dynamic vectors. This section will provide key information on mosquito life history traits important for understanding the remainder of the thesis. Basic knowledge of the mosquito life cycle will be provided before discussing anatomical features and biological pathways directly involved in WNV transmission. All processes that are discussed in detail will be directed towards the *Aedes* genus because all experimental procedures for this thesis were conducted on *Aedes* species only. See Appendix 2 for a basic introduction to mosquito anatomy at the different life stages.

1.4.1 Life Cycle

Mosquitoes have four distinct life stages: egg, larva, pupa and adult. Hatching from the egg is triggered by contact with water and the larval and pupae life stages are aquatic. They do not exhibit a terrestrial lifestyle until they have undergone complete metamorphosis into the adult winged form.

Egg Stage

The location where eggs are deposited is often species-dependant. Some produce a raft of eggs that float on top of the water's surface, others deposit eggs on moist organic

material near the edges of natural water bodies and some lay their eggs in artificial containers that can hold rainwater for longer than many natural locations (Bartlett-Healy et al. 2012). The shell of the egg surrounding the embryo is waterproof as well as porous so gas exchange can occur without dehydrating the embryo itself (Sota and Mogi 1992). Eggs are developed and stored by the female before fertilization occurs thus their structure contains a small pore (micropyle) that allows sperm to penetrate as the eggs pass down the oviduct during oviposition (Nation, 2008). The number of eggs per gonotrophic cycle varies between species with a range of 50-500 depending on each individual's resource situation (Nation 2008, Bartlett-Healy et al. 2012). The length of time eggs will remain without hatching also varies between species and is correlated with environmental temperature (Sota and Mogi, 1992). It can be a couple of days to over a month for eggs to hatch under normal conditions with some species producing eggs capable of withstanding desiccation for upwards of six months (Sota and Mogi, 1992).

Larval Stage

The larval stage follows hatching with the young, filter-feeding, worm-like mosquito larva exits the shell fully adapted to aquatic life. The larval stage is considered to play an indirect role in virus transmission as different environmental conditions can impact the size and nutritional reserves of the adults leading to a difference in vector competence (Alto et al. 2008). Tun-Lin et al. (2000) used field and laboratory studies to determine the effect that temperature and larval diet had on larval development time and emerging adult size. They found that fastest development occurred with increasing temperatures and increasing amounts of organic matter (food resources) in the larval

containers. They also noted that emerging adults were larger from high organic containers but decreased in size as their external temperature increases with the largest adults occurring from high organic and cooler environments. The nutritional reserves of the emerging adult was shown to influence vector competence as results from Alto et al. (2008) and Vaidyanathan et al. (2008) suggested significant increase in DENV transmission by smaller sized and nutritionally compromised females compared to larger, fed ones.

Pupal Stage

The "comma-shaped" pupa is the final aquatic stage of the mosquito life cycle as each individual undergoes complete metamorphosis. Although mosquito pupae can be very active, they do not feed in this stage. Once development of the adult is completed, the mosquito will take in oxygen in order to build up enough pressure to help break the pupal case along the dorsal ecdysial line (Clements, 1992) and the adult mosquito emerges.

Adult Stage

The winged adult is the stage most commonly associated with mosquitoes because adult females are considered human pests. Males are easily distinguishable from their female counterparts by the structure of their antennae. Male antennae are quite plumose because their setae are generally longer and more numerous compared to those found on females (Thielman and Hunter 2007).

As a source of energy both sexes utilise naturally occurring sugars from several sources like plants, rotting fruits, and homopteran honeydew (Gary and Foster 2006). Naturally occurring sugars provide the only nutrients for adult males as they have no protein requirement to produce sperm and therefore have evolved no mechanism for blood ingestion (Gary and Foster 2006). Most female mosquitoes are anautogenous, meaning they require proteins from a blood meal in order to produce eggs; however, some species are autogenous, meaning they can produce eggs during their first gonotrophic cycle without the need for a blood meal (Gary and Foster 2006).

1.4.2 Mating

Mosquitoes differ from most insects in their ability to mate while flying; males are stimulated by the flight tone of an active female (Gibson et al. 2010). If successful in finding a mate the male mosquito will orient himself venter-to-venter with her so that their external genitalia can be properly exposed and "hook" together (Clements, 1999). The male's aedeagus will become sealed within the female's vaginal wall allowing him to ejaculate a mixture of spermatozoa and accessory gland fluids into her bursa inseminalis (Clements, 1999). Successful insemination occurs when the male's spermatozoa migrate from the bursa inseminalis into one of her three spermathecal capsules where she will store it until her batch of eggs are ready to be fertilized (Clements, 1999). A detailed diagram of the female reproductive system can be found in Clements (1999). Studies on mosquito behaviour show that both males and females are sexually polygamous. A sexual reproduction study by Gwads and Craig (1970) noted that through forced copulation a single virgin male has the ability to fill two of the three

spermathecae in up to five virgin females and still have enough fluids to leave a trace amount of sperm in his sixth through ninth females. Choochote et al. (2001) determined that within their laboratory colony, a single female *Ae. albopictus* contained in a cage with ten males successfully mated with three of them in one hour. Boyer et al. (2011) estimated that on average an individual male mated with 9.5 females and a maximum of 14 over 7 days. This type of polygamous behaviour has most likely developed because it would increase the likelihood of a male passing on his genes and a female having enough sperm to fertilize her eggs (Gwards and Craig, 1970; Choochote et al. 2001)

1.4.3 Blood Feeding

Hematophagy is a complex behaviour involving many crucial steps in order to be successfully completed by a mosquito. The female must first find a blood meal host which involves a number of cues, such as temperature, carbon dioxide, olfactory stimuli, and visual stimuli (Kenney and Brault 2014). Even if a female locates a suitable blood meal host, she still has to land on the host, penetrate the epidermal layer with her proboscis, find a vessel containing blood, and ingest enough protein without being noticed. Aside from the proteins for egg development females are also able to gain energy from the blood meal as females fed solely on blood survived longer in the laboratory than those fed strictly on water (Kenney and Brault 2014).

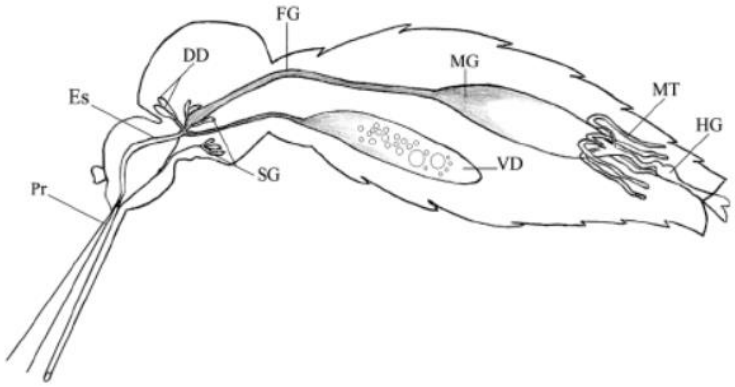

Figure 1.7- Alimentary canal of female *Aedes*: dorsal diverticulum (DD), foregut (FG), hindgut (HG), midgut (MD), Malpighian tubules (MT), esophagus (ES), proboscis (Pr), salivary glands (SG), and ventral diverticulum (VD) (crop). (Retrieved from "First isolation of microorganisms from the gut diverticulum of Aedes aegypti (Diptera: Culicidae): New perspectives for an insect-bacteria association" by Gusmao et al., 2007, in Mem Inst Oswaldo Cruz, Rio de Janeiro, 102 (8), p921)

1.4.4 Digestion

The alimentary canal of mosquitoes contains several different compartments, each playing a role in the digestion of nutrients within the mosquito (Figure 1.7). Upon successful ingestion of a fluid an innate response within the female dictates where the meal will end up. In nearly all species that have been examined when a sugar meal is ingested by the mosquito it is diverted into the ventral diverticulum or crop where "long term storage" of carbohydrates and starches occurs (Kenney and Brault 2014). Although hard to determine, it is generally accepted that carbohydrates and starches are maintained as liquids within the crop. They are slowly diverted to the anterior midgut for digestion, separate from where blood meal digestion occurs, so that contents of the sugar meal don't inhibit protein digestion (Nation, 2008). The crop epithelial cells are unspecialized in ultrastructure, interconnected by septate and spot desmosomes, and surrounded by an osmiophilic epicuticle layer (Nation, 2008).

When a blood meal is ingested it is always diverted directly into the posterior midgut (stomach) with digestion beginning almost immediately. Ingested food triggers the production of the peritrophic membrane which surrounds the food and aids in digestion, protects the midgut cell surface, acts as a barrier to pathogens, and prevents undigested materials from binding to the midgut surface (Nation, 2008). It prevents most pathogens from being able to infect the midgut cells because its pores are minute in size (20-30nm) and most pathogens are too large to pass through it (Kenney and Brault 2014). The midgut itself is composed of a basal lamina compacted within a thin set of muscle tissue that surrounds the inner single layer of epithelial cells (Clements, 1992; Nation, 2008). When a virus is ingested within a blood meal the first cells it must infect are the mosquito's midgut epithelium.

1.4.5 Escape Barriers

Fitness of an arbovirus primarily hinges on its ability to transmit out of one host body and into the next before the host dies and its cellular environment no longer facilitates survival. When a mosquito vector takes up virus within a blood meal it does not automatically result in its ability to transmit the virus out during its next blood meal. The virus must be present within the lumen of the mosquito salivary glands of the mosquito in order for it to be injected along with the salivary proteins during a blood feeding episode. A series of processes must successfully occur in order for the virus to be transmitted into and out of the mosquito vector.

An infectious blood meal is directed straight into the midgut upon entry into the alimentary canal (Figure 1.7) of the mosquito. From this point several possible scenarios

will occur as the virions attempt cellular reproduction within the vector (Perera et al. 2008; Clements, 2011; Kenny and Brault, 2014). If virus does not penetrate the host midgut epithelial cells and is excreted with other waste products of the mosquito then no infection is said to have occurred. This is partially due to a midgut infection barrier that can alter the infection rate of individuals from the same species (Anderson et al. 2010).

A midgut escape barrier has also been identified because virus may successfully infect midgut epithelium cells but progeny virions might not exit on the basolateral side of the cell (Kenny and Brault, 2014). An infection that results in virions exiting exteriorly from the midgut is said to be a disseminated infection and the presence of virions can be detected throughout tissues containing haemolymph including the legs and wings of the vector (Clements, 2011).

But in order to be fully capable of transmission via the next blood feeding event, virions must infect the cells of the salivary gland where free floating virions will be among the saliva protein solution that will be spit into the vertebrate host during blood feeding. Infection that can be detected in all tissues except those within the salivary glands constitutes the existence of a salivary gland entrance barrier (Kenny and Brault, 2014). It is also possible for virus to be detected within the salivary gland but not within the lumen where the salivary proteins are stored before feeding. Virus that is present in the gland but not within the lumen is suggestive of a salivary gland exit barrier (Kenney and Brault 2014).

1.5 Laboratory Methods of Detection

Numerous laboratory protocols are used in transmission studies. This section will highlight several commonly used protocols for detecting West Nile and other closely related viruses within the mosquito vector. The three main methods of detecting the presence of a virus within the mosquito vector are enzyme-linked immunosorbent assay (ELISA), TaqMan® reverse transcriptase polymerase chain reaction (RT-qPCR), and plaque assay.

1.5.1 ELISA

ELISA involves the use of antigen-antibody reactions monitored through a colour changing enzyme substrate reaction and is widely applied for detecting flaviviruses within human sera and individual mosquitoes (Gubler et al. 1984). Pathogen specific antibodies connected to a detection enzyme are designed to adhere with a corresponding pathogen. A final solution added to the mixture will undergo a colour change in the presence of the detection enzymes. If the corresponding pathogen is not present the detection enzymes would not have adhered to the sample and therefore no colour change would occur on the substrate.

The protocol is relatively cheap, compact, and easy to operate but does have a slight drawback as it is not sensitive to low virus concentrations and serological cross reactivity is known to occur between closely related viruses like those found within the Japanese encephalitis serogroup (Poidinger et al. 1996; Clements, 2011). A recent field study in Brazil detecting the presence of DENV in field caught mosquitoes highlights the benefits and drawbacks of ELISA as Martins et al. (2012) used the protocol to detect

DENV within their mosquito pools but required the use of PCR followed by gel electrophoresis to determine which strain of the virus was circulating within their samples.

1.5.2 RT-qPCR

Polymerase Chain Reaction (PCR) is a technique that targets a specific region of a genome through a series of reactions causing it to exponentially replicate or amplify. Because the genome of all viruses within genus *Flavivirus* are single stranded RNA, a reverse transcriptase (RT) enzyme must first be activated in order to create cDNA of the target sequence before the PCR reaction can take place (Lanciotti et al. 2000). Real time RT-qPCR is similar to traditional PCR except the amount of amplified product is measured after each round of replication as opposed to measuring the final end product.

An individual or group of mosquitoes is homogenized in a solution of media that facilitates virus survival and then a portion of the homogenate solution is used to extract the RNA from within it. That RNA is mixed with molecular reagents that facilitate the specific gene amplification and aid in detection of the reaction. TaqMan probes are hydrolysis probes that rely on the 5´–3´ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence, resulting in fluorescence emitted at the completion of the newly created strands (Sigma Life Science, 2008). The highest fluorescence would therefore be detected during the exponential phase of PCR when the greatest amount of product is created. The critical threshold value (C_T value) is the point at which light fluorescence is emitted considerably above the background level (Lanciotti et al. 2000; Shi et al. 2001). A lower C_T value

indicates that this critical fluorescence point was reached at an earlier amplification cycle than a larger C_T value. Therefore the amount of initial target RNA within a sample is inversely proportional to the C_T value it produces. If no target sequence is present, then no fluorescence will occur and thus no C_T value would be recorded. The extreme sensitivity of RT-qPCR for detection of WNV was exhibited by Turell et al. (2002) as they were able to detect WNV in infected mosquitoes held at room temperature for 14 days after dying.

They suggest that any sample run through their protocol with an estimated C_T value of <37 should be considered positive for the virus and >37 should be considered negative. In 2003 The Canadian National Steering Committee on West Nile Virus Surveillance established that the gold standard mosquito viral testing protocol be done according to Lanciotti et al. (2000). The committee altered their acceptance of a positive C_T value as <30 when detected by two different primers. Anything >30 must be re-extracted and retested. If the retest C_T value is <37 for both primers used, the sample should be considered positive and >37 for either primer should be considered negative.

1.5.3 Statistical analysis of RT-qPCR positives

RT-qPCR provides the ability to individually test or combine a large number of mosquitoes into a single sample and rapidly test for the virus among them. If the viral RNA is detected, a mathematical equation can be used to calculate the minimum rate of infection (MIR) within a pooled sample. Chow et al. (1998) calculated MIR as the number of positive pools/total number of mosquitoes tested*1000. The value provided is stated as the number of positives per 1000. The calculation can be somewhat misleading

with lower numbers of mosquitoes tested. In those cases, a positive mosquito pool can make the MIR seem quite large, when compared to the level of WNV activity.

1.5.4 Plaque Assay

The presence of a positive RT-qPCR sample does not indicate that an infectious mature virion was present. Due to the way a virion is produced within the cell or because of left-over RNA from a deactivated virion, RT-qPCR is unable to distinguish between infectious and non-infectious particles (Payne et al. 2006). This draw back to RT-qPCR can be corrected through plaque assay as this method of virus detection confirms the presence of and can quantify infectious particles within a sample. A portion of the initial mosquito homogenate is placed onto a culture of host cells and given sufficient time for virus particles to infect the cell layer. Because of the flaviviral dual host cycle, cell monolayers of either mammalian epithelial cells (e.g., African green monkey kidney cells, VERO E6 cells) or mosquito larval tissue cells (e.g., *Ae. albopictus* c6/36 cells) are most commonly used (White 1987; Payne et al. 2006). These cells are chosen because they propagate indefinitely, senesce at a slow rate, and unlike normal cells they do not initiate an interferon response when infected with virus (Desmyter et al. 1968; White 1987; Payne et al. 2006).

A mixture of agarose and media is placed on top of the cells which limits free floating virions to infecting only cells directly surrounding them; this creates a circular pattern of induced cell death known as a plaque. After a few days the cells are checked for cytopathic effect and the formation of plaques are visualised by staining the remaining living cells. Plaque assay can then be used to quantify the virus titer of a

sample and is expressed as plaque forming units per mL (pfu/mL) (Podlech et al. 2002). The formula used is $p/df*iv$ where *p* is the number of plaques counted, *df* is the dilution factor of the sample used for infection, and *iv* is the volume of sample added to the cells for infection (Podlech et al. 2002).

The biggest drawback of plaque assay is that it cannot be used for virus identification on its own, it must be coupled with a secondary test to identify the specific infectious agent and it is highly labour-intensive and time-consuming with several days needed for cells in culture to grow and for completion of the test itself (Clements 2011; Podlech et al. 2002). This was highlighted by Lothrop et al. (2012) as they were able to detect a WNV solution that had been exposed for 48h when using RT-qPCR but were unable to identify any infectious virions when testing the same sample in cell culture. Similar results were observed by Turell et al. (2002) while testing previously infected mosquitoes left in laboratory conditions 14 days after they had been killed. Their RT-qPCR readily detected WNV RNA from the dead individuals after two weeks, whereas cell culture techniques showed no signs of infectious particles.

1.6 Laboratory Infection Protocols

Laboratory studies involving transmission of an arbovirus must include a variety of repeatable control steps in order to maintain equal infection across all of the feeding mosquitoes as well as ensure the safety of all scientists conducting the research. This section will review the wide variety of methods that have been used in order to accurately and safely infect mosquitoes.

1.6.1 Oral Infection

A simple and natural method of oral infection is achieved by allowing female mosquitoes to feed on a previously infected vertebrate host. This type of oral infection was used by Turell et al. (2005) for their WNV vector competence study where they isolated virus from a wild caught infected crow, amplified the virus through cell culture, harvested the infected cell layer, and inoculated the quantified viral solution into leghorn chickens on which different mosquito populations were allowed to feed. Results of their infection rate were used to determine each species' vector competency for WNV. They even introduced uninfected leghorn chickens to the previously infected mosquitoes to determine if the species was able to transmit and infect the naive chickens. Feeding laboratory mosquitoes on live animals has several benefits as the process involves many of the natural cues essential for surviving in nature, the blood itself would contain a more natural balance of resources, and would be consistently held at the proper temperature (Kasap et al. 2003).

However, there are numerous bioethical rules regarding the animal's welfare that must be considered when working within a laboratory setting which require specific care, housing, and handling of the animals themselves (Luo 2014). These types of requirements can lead to complications when they result in more than one host being used for a single experiment. Different biological factors within each individual host can lead to an unequal infection dose when more than one is used to infect a large number of mosquitoes.

Alternative methods for infection have been developed because not all labs are permitted to work with infected vertebrate species. Blood extracted from vertebrates can

be mixed with virus and delivered to females through something as simple as soaking a cotton pad or through a variety of specially designed membrane and suspension feeders (Rosen 1987; Mourya et al. 2001; Joshi et al. 2002; Clements, 2011). It has become common for laboratory studies to use one of the several varieties of similar functioning membrane feeders in order to orally infect mosquitoes. The basic design of the Hemotek® membrane feeder (Discovery Workshops, Accrington, UK) is a heated well that gets covered by either a wax film or vertebrate skin that holds the blood inside but allows it to be exposed to mosquitoes (Goddard et al. 2002). Luo (2014) created a unique membrane feeder similar in functionality to the Hemotek feeder but slightly different in its ability to use multiple wells simultaneously. He detected no significant difference in feeding from their apparatus versus exposure to infected mice.

Unfortunately the protocols listed above are only viable for infection of female mosquitoes since males do not take blood meals. Aragão (1929) developed a repeatable protocol for infection of males by mixing YFV with a sugar solution. In order to achieve ingestion of virus, Aragão (1929) dipped cotton into an infectious solution of rhesus monkey blood and honey and placed the soaked pad in a cage allowing starved male mosquitoes to feed on the solution. Males were collected and homogenized together in a solution that was then injected into a rhesus monkey. Infection was confirmed in two trials in which monkeys showed symptoms of yellow fever. Lothrop et al. (2012) modified this method of infection using WNV mixed with sugar and placed onto cotton pads. They conducted experiments on groups of naturally caught *Cx. tarsalis* with each group being exposed for 48h to an original virus titer ranging from 10^1-10^7 pfu/mL. Only

9 of 27 individuals fed on the 10^7 titer tested positive for virus; all other titers produced no detectable infections.

1.6.2 Mechanical Infection

A commonly used protocol for infecting mosquitoes is intrathoracic inoculation (ITI). The idea of infecting mosquitoes by some form of injection has been used for many decades. Chamberlain and Sudia (1961) stabbed an infected chick with a pin and subsequently stabbed a mosquito resulting in transmission of EEE. The technique was modernized in Rosen and Gubler (1974) who used compressed air to force an infectious solution through a tube, into a fine needle, and eventually into the individual mosquito. ITI results in virus inoculated directly into the hemolymph because the needle is inserted just above where the thorax and the head meet (Rosen and Gubler 1974). ITI is highly regarded for the ease in which a large number of mosquitoes can be infected and the speed of disseminated infection (Rosen and Gubler 1974). ITI bypasses the midgut escape barrier and decreases the normal extrinsic incubation period to achieve infection within the salivary glands. This decreased time allows researchers to conduct dissemination and transmission experiments on a larger number of individuals because most would succumb to death before the virus was able to disseminate into specific organs throughout the body (Turell et al. 2005).

The innate immune response of mosquitoes is well documented as the insect vector is equipped with processes that protect it from pathogens, parasites, and injury (Blair 2011; Kenney and Brault 2014). Han et al. (1999) discovered that within 30 minutes of sustaining an injury at least six distinct hemolymph polypeptides were

consistently produced after the cuticle wall had been ruptured. Immune responses come at a cost to the individual as there is an increased physiological and energetic stress when the pathways are activated (Ardia et al. 2012). ITI is well documented for its ability to evade the midgut barriers within a vector but it will also create additional stress as both the puncture wound and viral infection will trigger innate immune responses and could influence downstream transmission pathways.

1.6.3 Viral Titer

The concentration of the infectious solution plays a direct role in the rate at which the cells of the mosquito vector become infected with a virus (Perera et al. 2008). To accurately infect their population, laboratory studies use a variety of cell culture and viral detection techniques in order to harvest and quantify a stock of virus solution with a known concentration of virions. The greater number of active virus particles within a solution the higher the chance that one will successfully enter the cell and begin its amplification process (Perera et al. 2008).

Investigation of several North American avian reservoir species by Komar et al. (2003) determined that the WNV blood titer among 25 species of birds varied greatly ($10^{1.7}$pfu/mL to $10^{12.1}$pfu/mL). There is evidence that some mosquito species can become infected when feeding on a blood titer as low as $10^{3.0}$pfu/mL (Goddard et al. 2002). But Komar et al. (2003) suggest most mosquitoes feeding on birds with a virus titer of $<10^{5.0}$pfu/mL would probably not establish a transmission cycle; thus, they proposed a minimum host blood titer of $10^{5.0}$pfu/mL for infection which agrees with previous titer requirements suggested by Turell et al. (2001) for *Cx. pipiens* and by Sardelis et al.

(2001) for *Cx. quinquefasciatus*. The infectious dose concentration provided to mosquito vectors is very important as an increase in titer can produce a significantly higher rate of infection within mosquitoes of the same species. This type of discrepancy was experienced by Turell et al. (2001) when chickens inoculated days earlier developed a large gap in blood titers ($10^{5.2\pm0.3}$ and $10^{7.2\pm0.3}$ pfu/ml of blood) that increased the WNV infection rate of *Cx. pipiens* by 64%, *Ae. sollicitans* by 59%, and *Ae. vexans* by 46%. Anderson et al. (2010) found a significant infection rate difference among *Cx. quinquefasciatus* when mosquitoes were infected with a low dose of $10^{5.9}$pfu/mL as opposed to a high dose of $10^{7.0}$pfu/mL.

1.7 Secondary WNV Transmission

There are several modes of transmission in which an arbovirus can successfully infect a new host without the need for blood feeding. Vertical transmission is the direct transmission of a pathogen with no intermediate host. It is considered by many to be secondary in maintaining the natural survival of an arbovirus because the rate at which it occurs is thought to be much lower than primary transmission in nature (Kenney and Brault 2014). This section will highlight two modes of vertical transmission and the debate associated with their naturally occurring rates and their ability to influence virus survival.

1.7.1 Transovarial and Transovum Transmission

Virus that has disseminated throughout the hemolymph of a female mosquito can also disseminate into her reproductive tract where the production of eggs and the storage of male spermatozoa takes place (Mourya et al. 2001; Kenny and Brault, 2014). At this point there are two different moments when the virus can enter the egg and infect the future progeny. Transovarial transmission describes virus that invades the oocyte within the ovary (Clements, 2011). Transovum transmission designates virus within spermatozoa fluid that enters the egg at the moment of fertilization during oviposition (Clements, 2011). Although there is a distinct difference between these two processes, the literature rarely establishes the exact moment when the progeny and virus come into contact (Rosen, 1987; Mourya et al. 2001; Clements, 2011). From this point on transovarial transmission (TOT) will be used to describe the infection of progeny from an infected female regardless of which transmission mode took place.

Although TOT of a flavivirus in its mosquito vector was hypothesized by many scientists working with YFV and DENV, it was not demonstrated in the laboratory until Coz et al. (1976) reared infected *Ae. aegypti* progeny from Koutango virus infected females. Over the next few decades, laboratory experiments demonstrated that TOT is a component of many flavivirus transmission cycles including SLEV (Hardy et al. 1980), YFV (Aitken et al. 1979), DENV (Rosen, 1997), JEV (Rosen et al. 1989), MVEV (Kay and Carley 1980) and WNV (Baqar et al. 1993; Dohm et al. 2002; Thongrungkiat et al. 2012). In the field, naturally occurring TOT can be detected by testing for virus in larvae or non-biting male mosquitoes because there is no likely method that an infection could have occurred other than through TOT (Clements, 2011). Laboratory studies on La

Crosse virus and DENV ruled out male infection due to copulation with infected females (Thompson and Beaty 1978; Mourya et al. 2001). Numerous studies have provided clear evidence of several flaviviruses in wild *Ae. albopictus* populations at both the larval and adult male life stages (Mourya et al. 2001; Joshi et al. 2002; Mulyatno et al. 2012). Mourya et al. (2001) reared the offspring of infected *Ae. albopictus* and detected that vertically infected females were capable of transmitting DENV into the blood meal provided to them. This was the first evidence that vertically infected females were capable of transmitting virus to a new host during a blood meal. A few years later, Anderson and Main (2006) demonstrated that vertically acquired WNV infection could be horizontally transmitted. They held *Cx. pipiens* progeny from infected mothers under winter conditions and eventually allowed them to feed on an uninfected hamster that died eight days later of WNV infection.

Initially it was hypothesized that TOT facilitated a virus' survival within the mosquito vector during extended environmental conditions that are unfavourable for the mosquito vector (Anderson et al. 2006). The potential for a virus to be passed along to a female's unborn progeny would explain how a virus can survive in temperate climates during winter months and during periods of prolonged drought where mosquito populations become dormant yet virus transmission occurs as soon as conditions change (Anderson et al. 2006; Mulyatno et al. 2012). Transovarial transmission followed by horizontal transmission into the primary transmission cycle would also explain how viruses can survive in urban areas for extended periods without being detected by an enzootic or human epidemic (Martins et al. 2012)

1.7.2 Transovarial Transmission Rates

Although there are several occasions where the rate of TOT is quite high (Joshi et al. 2002; Anderson et al. 2008; and Mulyanto et al. 2012) overall the estimated rate at which it occurs does not merit significant concern for this type of virus transmission. Adams and Boots (2010) reviewed DENV TOT rates found in the literature and put them into a mathematical model to help understand the role of this transmission on DENV persistence. They noted that most laboratory experiments suggest a relatively low TOT efficiency of 1–4% and at these rates TOT has no more than a weak influence on DENV persistence.

Infection studies by Mourya et al. (2001) and Anderson et al. (2008) both found evidence that could lead to these low TOT rates. Both research groups infected females and provided more than one blood meal allowing eggs of different gonotrophic cycles to be deposited before testing the female and rearing the progeny. Mourya et al. (2001) found 83% of their positive DENV larval pools were hatched from the 2^{nd} – 4^{th} gonotrophic cycles in *Aedes* mosquitoes. Anderson et al. (2008) didn't detect TOT of WNV until the second gonotrophic cycle in *Culex* mosquitoes. An explanation for these results could be found in the method of TOT as production and fertilization of the eggs could be completed well before the virus escaped the midgut. This creates the possibility that a female could be infected with virus but her first batch of eggs has no potential for infection. Inclusion of progeny from the first gonotrophic cycle after an infectious blood meal could be under-estimating the actual rate of TOT (Rosen, 1988; Mourya et al. 2001; Anderson et al. 2008).

There is also a flaw in the calculation of MIR which may reduce the suggested rate of TOT. Martins et al. (2012) noted that within 1 of their 3 pooled samples testing positive for DENV, there were actually two different strains of the virus present. The detection of a second DENV strain means that either the same mosquito was infected with two strains (unlikely according to them) or a second mosquito was in fact positive for the second strain. The MIR equation does not account for the possibility that more than one individual within a pool can be infected with the virus, which indicates that the current calculation for MIR is actually underestimating infection rates of pooled mosquitoes (Gu et al. 2008). Therefore this assumption must be taken into account when analysing pooled mosquito data using MIR and the only way to eliminate this possibility is to individually test mosquitoes for virus but would only make sense when testing a smaller sample size.

There is evidence that suggests TOT could be occurring at a much higher rate in nature than what laboratory studies propose (Angel and Joshi, 2008; Mulyatno et al. 2012). Angel and Joshi (2008) found a DENV TOT rate of 18.7% (no. positives/ no. tested) in a population of *Ae. albopictus* and Mulyatno et al. (2012) noted that adults reared in lab consistently had a lower MIR than those from nature. Difficulty arises because adult females who test positive in nature for a virus are assumed to have become infected via primary transmission. Only larvae or adult males collected in nature provide evidence of natural TOT and therefore the rate at which this transmission cycle is occurring is quite hard to decipher. There is also evidence to suggest that secondary transmission in natural populations may not occur most often during unfavourable

conditions as Mulyanto et al. (2012) found higher TOT MIR during rainy seasons (23.89) compared to the dry seasons (11.79) in an Indonesian population of *Ae. aegypti.*

1.7.3 Venereal Transmission

The presence of an infected adult male mosquito shows evidence of naturally occurring TOT but also limits the role that it plays in virus survival. The sex of the adult mosquito is predetermined in a similar fashion as it is in humans (Clements, 1992; Nation, 2008). Therefore half of all vertically infected offspring are expected to be male, limiting the ability of TOT to jump back into the primary transmission cycle since males do not take a blood meal.

Venereal transmission (VT) is a mode of vertical transmission where a pathogen carried in the spermatozoa fluid of vertically infected males enters females during mating, resulting in their infection (Clements, 2011). The rate of VT is loosely defined as the proportion of a female mosquito population infected by vertically infected males through copulation (Clements, 2011). For obvious reasons this type of transmission can be quite difficult to detect in natural populations. Because of this difficulty to establish a naturally occurring rate the impact of VT is of course linked to the natural method by which males become infected, TOT. Low observed rates of TOT is likely to be the cause behind the lack of VT literature (Knell and Webberley 2004). They identified VT of only 3 flaviviruses, which to them was a startling low number of studies conducted on this epidemiologically important genus. Since their publication, only an additional 2 laboratory studies have provided evidence of *Flavivirus* VT. The process has been

identified in different vector species for DENV (Rosen, 1987), SLEV (Shroyer, 1990), JEV (Rosen et al. 1989), CHKV (Mavale et al. 2010), and WNV (Reisen et al. 2006).

Rosen (1987) infected male *Ae. albopictus* with one of the 4 DENV serotypes and introduced them to uninfected virgin females either 7 or 14 days later. All females seen mating with infected males were separated from the infected males and tested 14 days later for VT using ELISA. In total, 158 females mated with 7 day infected males and 135 with 14 day. Rosen (1987) identified VT rates in females ranging from 12, 2, 0, and 3% for the each of the 4 serotypes respectively with 7 day males and 64, 17, 16, and 15% with 14 day males.

A similar study by Reisen et al. (2006) tested the WNV VT rate within the Californian vector *Cx. tarsalis.* They infected males and introduced uninfected virgin females but had trouble successfully mating the sexes. They bypassed this obstacle by force mating infected males with anesthetized uninfected females. This method provided them with 21 successfully mated females which they then held either overnight or 3-5 days before testing. WNV RNA was detected in 2/3 and 1/18 females, with 1 of them containing enough infectious particles to be identified in plaque assay.

Primary Objectives

The specific aim of this research was to investigate vertical transmission of WNV under natural and laboratory conditions to provide a better understanding of the role this type of transmission plays in the survival of the virus.

To reach these objectives, field surveillance collections were used to investigate naturally occurring TOT during the 2012 and 2013 seasons. The results of this study could have an impact on the debate over the importance of secondary WNV transmission and its role in virus survival.

The original goal of the laboratory experiments was to investigate if the initial rate of WNV TOT differed from the rates observed in progeny from subsequent gonotrophic cycles. These initial experiments were designed with the assumption that females would readily feed on infectious blood meals within the laboratory. This was met with frustration as very few females successfully engorged on infectious blood which resulted in an insufficient sample size for conducting the experiments. While trying to increase the successful feeding rate, an alternative infectious solution was created to entice a larger group of individuals to orally ingest the virus. The preliminary experiments of the artificial solution showed promise as the successful feeding rate jumped from approximately 5% to 90% while using this easily repeatable new protocol. The next objective was to determine if the new protocol could be used to assess the vector competency of mosquitoes by producing infection, dissemination, and transmission results that were similar to the more common oral infection protocols involving blood feeding. If the protocol produced repeatable results that were comparable

to those observed in previous literature, this new method could be implemented to investigate the vector competency of mosquitoes.

Because the protocol does not involve the blood feeding behaviour of female mosquitoes, it could also be used to orally infect male *Ae. albopictus* mosquitoes and assess their ability to venereally transmit the virus to uninfected females. The ability of *Ae. albopictus* to transmit WNV venereally would provide further information about the potential impact of vertical transmission on virus survival. It would also justify the need for VT research on the other flaviviruses known to be transmitted by the vector because they too could be surviving through this secondary mode.

Chapter Two

Difficulties associated with the field detection of vertically transmitted West Nile Virus in Ontario during the 2012 and 2013 field seasons.

2.1 Abstract

The rate at which West Nile Virus (WNV) is vertically transmitted to offspring is highly variable. It has been suggested that a transovarial or transovum transmission (TOT) cycle would facilitate the survival of the virus during extended periods of unfavourable conditions. However, recent studies have suggested this type of transmission may be most prevalent during peak seasons of virus activity and not limited to occur only during specific environmental conditions.

In Ontario, 2012 was the second highest year for WNV activity in mosquitoes since surveillance started in 2002. Males collected in CO_2-baited CDC light traps as part of Ontario's 2012 province-wide mosquito surveillance program were tested for naturally occurring TOT of WNV. In 2013 wild caught eggs, larvae, pupae, adult males and gravid females were collected from sites in the Niagara Region of Ontario between June and September and were tested for the presence of naturally occurring WNV TOT. The majority of the untested 2012 samples had already been discarded by the time this research started but 89 males from 7 different traps were recovered from the remaining 2012 collection samples. The 2013 Niagara region field collections resulted in the testing of 1023 individuals across 9 different WNV vector species. None of the specimens tested yielded any evidence of WNV TOT. However, a single wild-caught gravid female *Culex pipiens* tested positive for WNV during the study period. Her 270 larval offspring and remaining unhatched eggs were negative for the presence of viral RNA. Although this is not conclusive evidence that TOT of WNV was not occurring in Niagara during the sampling period, it does highlight many of the difficulties in testing for this type of virus transmission.

2.2 Introduction

West Nile Virus (WNV) was first detected in Windsor Ontario from an infected bird collected in early August 2001 (Drebot et al. 2003). Prior to WNV, flaviviruses were not of major concern in Canada. In 2002 the virus was detected in Ontario, Quebec, Manitoba, Saskatchewan, and Nova Scotia (Drebot et al. 2003). 2005 was the largest epidemic of human cases across Canada.

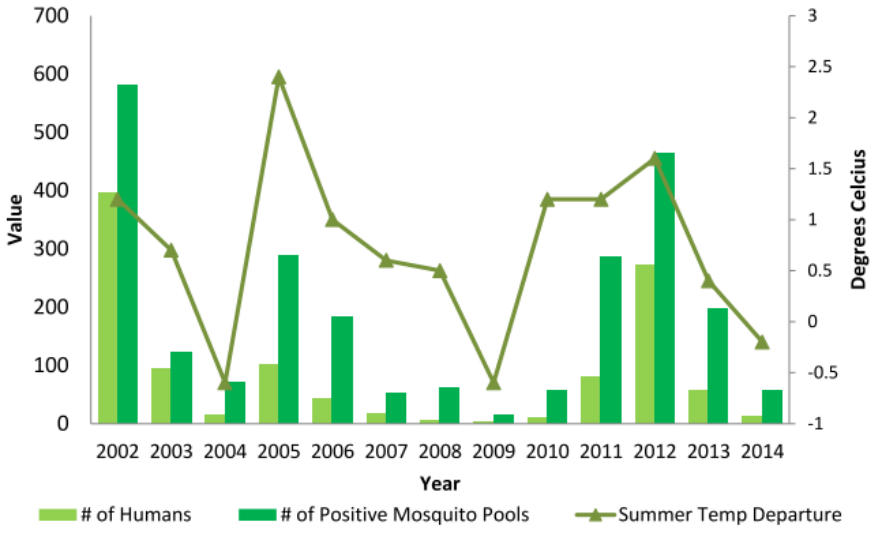

Figure 2.1- Number of reported West Nile Virus human cases and positive mosquito pools; and average summer temperature departures: Ontario, 2002–14 (Retrieved from Public Health Ontario, 2014)

The detection of virus in back to back seasons was not expected as many believed the temperate climate's winter conditions would limit the virus' survival and annual circulation (Drebot et al. 2003; Anderson and Main 2006). In Ontario, activity of WNV in the mosquito population has been tracked since 2002. Aside from the first epidemic year, the largest number of positive mosquito pools occurred in 2012 (464 positive pools) with drop off in activity during the 4th highest 2013 season (198 positive pools) (Figure 2.1) (Public Health Ontario 2012; Public Health Ontario 2013). In 2012 and 2013 the

majority of positive mosquito pools were reported in the Golden Horseshoe area, as well as southwestern and southeastern Ontario (Public Health Ontario 2012; Public Health Ontario 2013).

The virus has been isolated in nature from over 65 different species across North America (CDC, 2012) with over 25 of these shown in laboratory studies to be competent vectors (Turell et al. 2005). The 464 positive pools in 2012 consisted of 7 different mosquito taxonomic groupings, probably representing 8 species: *Cx. pipiens/restuans, Cx. salinarius, Aedes vexans, Ochlerotatus triseriatus, Oc. trivittatus, Anopheles punctipennis,* and *Oc. japonicus* (Public Health Ontario, 2012). In 2013 there were 5 taxonomic groupings that tested positive, probably representing 7 species: *Cx. pipiens/restuans, Ae. vexans, Oc. triseriatus/hendersoni, Anopheles punctipennis,* and *Oc. japonicus* (Public Health Ontario, 2013). In Ontario, *Cx. pipiens/restuans* has been identified as the main WNV vectors due to the large numbers of positives collected and a shift in their diet at the end of the summer season from mostly avian hosts to a broader range that includes humans (Drebot et al. 2003). These two species are grouped together because the adults are quite difficult to tell apart aside from a small circular patch of white scales on the thoracic region that is regularly rubbed off when caught in CDC light traps.

Transovarial or transovum transmission (TOT) is the passing of a pathogen from an infected female mosquito to her progeny, resulting in successful infection of the progeny. TOT was hypothesised to explain the phenomenon of arbovirus detection in wild caught male mosquitoes and from isolated locations with extended periods of unfavourable environmental conditions (Aitken et al. 1979). Theoretically, for a virus to

survive winter it must either be maintained in a constant horizontal transmission cycle or be sustained for a long period within a single host. Since mosquitoes aren't biting at a constant rate throughout the winter months, it seems unlikely that a continuous horizontal transmission cycle will occur. Initial hypotheses such as this lead to the widely accepted theory that TOT is somewhat of a security measure that the virus uses to increase the probability it will survive into another host (Aitken et al. 1979; Dohm et al. 2002).

Numerous flaviviruses have demonstrated TOT in laboratory experiments, including St. Louis Encephalitis Virus (SLEV) (Hardy et al. 1980), Yellow Fever Virus (YFV) (Aitken et al. 1979), Dengue Virus (DENV) (Rosen, 1997), Japanese Encephalitis Virus (JEV) (Rosen et al. 1989), Murray Valley Encephalitis Virus (MVEV) (Kay and Carley 1980) and West Nile Virus (WNV) (Baqar et al. 1993; Dohm et al. 2002). Evidence of naturally occurring TOT can be provided by any virus-positive wild caught larva, adult male, or previously unfed adult female. It is highly unlikely that male mosquitoes have acquired infection from any source other than their infected mothers and larvae reared in contact with virus rarely showed infection at the adult stages (Collins 1963; Thompson and Beaty 1978; Mourya et al. 2001).

Miller et al. (2000) were the first to discover WNV from male mosquitoes and others since have discovered infected wild males (Andreadis et al. 2001; Goddard et al. 2002; Unlu et al. 2010) and infected adults reared from wild collected eggs and larval stages (Reisen et al. 2006). Natural WNV TOT has been detected in over 15 different species including *Cx. pipiens, Cx. restuans, Cx. quinquefasciatus, Cx. salinarius, An. quadrimaculatus, Ae. albopictus, Ae. vexans,* and *Ae. triseriatus* (Andreadis et al. 2001; Goddard et al. 2002; Unlu et al. 2010).

In North America field rates of naturally occurring TOT are most often estimated using individuals collected from overwintering populations (Goddard et al. 2003; Anderson and Main 2006; Unlu et al. 2010). Recent field evidence suggests TOT might not follow the predicted transmission pattern as Mulyanto et al. (2012) conducted surveillance on DENV in Indonesia from 2008-2011 and determined that the virus was vertically transmitted most often during peak virus activity in the most favourable environmental conditions. Therefore estimations on the naturally occurring rate of TOT should include surveillance conducted on natural populations during peak virus activity as well as those experiencing unfavourable conditions.

This study was designed to test mosquitoes from collections across Ontario and Niagara Region for naturally occurring TOT of WNV during the 2012 and 2013 seasons. If it can be shown that WNV undergoes TOT within Ontario vectors it is possible that current protocols for virus surveillance are missing potential positives and therefore, virus activity in an area is being underestimated.

Identification of WNV in wild caught eggs, larvae, pupae, adult males and reared females all suggest that infection was acquired via TOT. Therefore this study looked at males collected from stored trap collections that had been set across Ontario during the 2012 season and in wild caught individuals from traps set throughout the Niagara Region during the 2013 field season.

2.3 Methods

2.3.1 Trapping

Trapping for Public Health Ontario's mosquito surveillance program is carried out by each individual health unit. During the 2012 WNV surveillance program, Entomogen Inc. was awarded several health unit contracts where they were to sort female WNV vectors into pools and test them for the presence of WNV. Untested individuals, including males, were stored at -20^{o}C until the end of the season. The samples of males for the current study were recovered from samples that had not yet been discarded; samples were resorted and any males within them were removed and tested for the presence of WNV.

Field collected samples were obtained between the second week of June until the first week of September, 2013. All samples were collected throughout the Niagara Region of Ontario, Canada. Each week 7 out of 12 collection sites encompassing the municipalities of St. Catharines, Thorold, and Welland were randomly selected for trapping. Traps were set at each of the 7 locations on Monday mornings (except holidays when trap set-up was moved to Tuesdays) and collected 24 hrs later. Two different trap types were set at each collection site. CDC light traps (Model 1012, John W. Hock Company, Gainsville, Florida, USA) baited with CO_2 to capture adults were hung in trees at shoulder height and gravid traps (model 1712, John W. Hock Company, Gainsville, Florida, USA) baited with stagnant water were used to capture gravid females. Stagnant water for each gravid trap consisted of 4L of tap water incubated for 72 hrs with 4g brewer's yeast and 20g (w/v) dried leaf matter mixed in.

At one of the trap locations, 2 used tires were set up as an artificial collection site for larvae and eggs. The tires were baited with stagnant water and placed in the shade to minimize water evaporation. A paper towel ovistrip was also provided so that *Aedes* eggs could be collected. Only natural rainfall throughout the season was needed to maintain sufficient water level for collection of mosquito larvae and eggs. Additional larvae and eggs were frequently collected from standing natural and artificial containers using a standard dipping ladle, a turkey baster, or a plastic 5mL plastic transfer pipette.

2.3.2 Mosquito Identification

Adult mosquitoes collected in the wild were sexed and females were identified to species using the photographic key in Thielman and Hunter (2007) while larvae were identified using the key found in Wood et al. (1979).

2.3.3 Mosquito Collection

Males from the 2012 WNV surveillance traps were sorted in groups of 5 individuals from the same location and placed into 1.5mL microfuge tubes for testing. Males collected in 2013 were placed individually into tubes.

Gravid trap collections were taken into the laboratory where females were individually separated (using an aspirator gun) and placed into 50mL conical tubes filled with 10mL of water to induce oviposition and a paper towel substrate to aid in climbing off the water's surface. Only females that successfully oviposited eggs were screened for the presence of WNV with all unsuccessful females discarded. Larvae were reared for species identification and kept only until testing for virus was completed. Because WNV

is considered to be a containment level 3 virus by PHAC any offspring from an infected mother were immediately pooled in tubes by instar and placed in a -20^{o}C freezer.

Wild caught larvae were identified and placed into 1.5mL microfuge tubes according to species and trap location. No sample containing only larvae exceeded 25 individuals within a single testing pool.

Several wild collected *Culex* egg rafts were split in half at the laboratory. Half were placed into a 1.5mL microfuge tube for testing while the other half were placed in plastic containers filled with stagnant water and reared for identification. A subset of reared larvae were placed into a 1.5mL microfuge tube for testing while the remaining individuals were reared to adults. Ovistrips collected with wild *Aedes* eggs were placed into plastic containers filled with stagnant water and reared for identification. A subset of reared larvae were placed into a 1.5mL microfuge tube for viral testing.

2.3.4 WNV Testing

Viral testing was done according to the PHAC gold standard national guidelines and followed the protocols established by Lanciotti et al. (2000) with some minor changes.

The BA-1 media was changed to supplemented Dulbecco's Modified Eagle's Medium (DMEM) in order to reduce costs. Experimental evidence showed no significant difference between the two media types, in regards to loss of viral titer from repetitive freeze/thaws (Bryan Giordano, 2014, personal communication). An additional benefit of using this DMEM recipe is that these reagents are also used for cell culture and plaque assay procedures when growing West Nile virus.

The kit used for testing was also changed to iScript One-Step (Bio-Rad Cat. #: 170-8895) as opposed to the QIAamp viral RNA kit (QIAGEN, Valencia, Calif.). In addition to changing kits the suggested 50µL (5µL RNA and 45µL master mix) final volume used during RT-qPCR was also reduced to a final volume of 25µL (5µL RNA and 20µL master mix) per reaction. Experimental evidence showed that substituting the kits and reduction of the final volume had no significant impact on detection of a positive control in a series of dilutions (Larissa Barelli, 2014, personal communication). Full protocols including media and reagent recipes for mosquito dissection, homogenization, RNA extraction, and RT-qPCR can be found in Appendix 3.

2.3.5 Minimum Infection Rate

The proportion of infected mosquito pool samples was estimated using minimum infection rate, MIR, as is standard for the West Nile Virus Surveillance program in Canada. The samples being tested are only a small subset of the much larger mosquito populations in nature. To account for this, MIR is calculated as it takes into account the small sample size being tested and alters it to better represent the much larger wild population (Chow et al. 1998). It is calculated as the number of positive pools/total number of mosquitoes tested*1000.

2.4 Results

A total of 89 males were collected out of traps set throughout Ontario during the 2012 season. 18 pools of 5 males maximum all tested negative for WNV. Identification to the species level was difficult within the samples as many individuals were damaged during sorting; therefore tubes contained a mixture of different species (Table 2.1).

The 2013 field collections yielded 1,023 mosquitoes and approximately 900 eggs tested for TOT of WNV (Table 2.2). 183 gravid females from 9 different WNV vector species were collected in gravid traps and placed into conical tubes for oviposition in the laboratory: *Cx. pipiens, Cx. tarsalis, Oc. japonicus, Oc. triseriatus, Oc. triseriatus, Ae. trivittatus, An. punctipennis,* and *An. quadrimaculatus*. Eggs from 90 of these females were successfully oviposited in their conical tube and 1 female *Cx. pipiens* tested positive for WNV. Offspring from the female were immediately separated into 3 instar pools (1^{st} 113, 2^{nd} 143, 3^{rd} 14) and screened for virus. All three pools tested negative for WNV.

Table 2.1- Summary of males collected during the Ontario WNV surveillance program in 2012 were tested for TOT of WNV. Males were collected from 7 traps set across Ontario as part of the province's 2012 WNV surveillance program.

Trap Id	No. Tested (no. pools)	Positive Pools
Alderville	44 (9)	0
Christian Island	10 (2)	0
Thunder Bay	13 (3)	0
Burlington	9 (2)	0
Hearst	4 (1)	0
Hearst	4 (1)	0
Kapuskasing	5 (1)	0
Total	**89 (18)**	**0**

Table 2.2 Summary of 2013 samples tested for TOT of WNV. All samples were collected within the Niagara Region of Ontario. Samples were tested by qRT-PCR following the testing protocol of Lanciotti et al. (2000). *270 tested larvae were the offspring of the female collected in a gravid trap that tested positive for WNV.

Species Tested	Gravid females providing eggs	Females in light trap of positive gravid	Wild egg rafts	Wild larvae	larvae from wild eggs	Males	Total
Cx. pipiens	51	25		20	270		366
Cx. restuans	14	15	3.5 (~900 eggs)	120	210		359 (+ 900 eggs)
Cx. tarsalis	1						1
Cx. erraticus	1						1
Oc. japonicus	6			140			146
Oc. triseriatus	1						1
Ae. trivittatus	1						1
An. punctipennis	2						2
Ae. quadrimaculatus	1	1					2
Aedes spp.		8					8
Culex spp.	11			10	110	4	135
Anopheles spp.	1						1
Total	90	49	3.5 (~900 eggs)	290	590	4	1023 (+ 900 eggs)
Pools Tested	90	4	7	14	35	2	152
Positive	1	0	0	0	0	0	1
MIR	11/1000	0	0	0	0	0	1/1000

2.5 Discussion

TOT of WNV was first discovered in adult male mosquitoes by Miller et al. (2000) in Kenya. Between 1997 and 1998 they tested 26 wild caught adult males in 11 different pooled samples and detected WNV from a sample containing 4 *Culex univittatus* complex males. Although TOT was not detected in any of the male samples in the current thesis, this does not rule out the possibility that WNV is maintained in Ontario via a naturally occurring TOT cycle. This lack of TOT detection should be used to highlight the many difficulties associated with detecting this type of transmission.

The major difficulty when testing for natural TOT is the “Blind” approach field studies must take as there is no definitive way to determine if the mother of wild caught individuals was ever in contact with WNV. Only 198 of 8,642 mosquito pools tested positive (2.3%) during the 2013 Ontario surveillance program (Public Health Agency of Canada, 2014). Mosquito pools have an upper limit of 50 individuals per pool which means a maximum of 432,100 individuals were tested. The 198 positive pools would have contained between 198 (1 per tube) and 9,900 (50 per tube) individuals. These values put into perspective the vast population size of mosquitoes and how difficult it can be to assess a naturally occurring transmission rate.

Even if the mother were infected, within each individual mosquito the virus must disseminate out of the midgut and enter the egg prior to oviposition. Differences in WNV transmission rates among populations of the same species are seen regularly in vector competence studies (Turell et al. 2005) and can be related to the way in which the virus replicates throughout the mosquito vector. There are several biological barriers prohibiting infection of the important cells involved in transmission that lead to differences in transmission rates between species and between individuals of the same species (Anderson et al. 2010). A difference in virus concentration of the blood meal, the extrinsic incubation period, and which gonotrophic cycle the offspring were from, have all been shown to influence the rate of virus transmission (Rosen, 1988; Dohm et al., 2002). Studies involving wild caught mosquitoes are therefore limited by these influences within each individual.

The possibility does exist that an adult female was infected via TOT but there is no conclusive method to test for this without her being reared in a laboratory. This

automatically decreases the probability of detection in half as only adult males represent naturally occurring TOT. Testing only wild caught adult males eliminates time spent rearing larvae within the lab but can be quite difficult as males are generally not collected in large numbers in CDC light traps. The current study considered these difficulties and took several approaches to limit their negative impact and increase the likelihood of detecting natural TOT of WNV.

Adult males were selected from traps set during the second highest year on record for WNV activity within Ontario (Public Health Agency of Ontario, 2013) in accordance with recent literature that suggested TOT rates peak with virus activity (Mulyatno et al. 2012).

Approximately 900 eggs, 610 wild larvae, and 4 males were tested for WNV during the 2013 sampling (Table 2.2). All of these came back negative, but there was no way to determine if the mother of the offspring was ever in contact with the virus to begin with. To limit this type of "unknown" testing, 189 gravid females were collected and placed into conical tubes where only the 90 females who successfully provided eggs before dying were identified to species and tested for the virus. The main vector of WNV in Canada, *Cx. pipiens/restuans*, made up 72% (65/90) of the gravid females that provided eggs and one of these tested positive during epi-week 29 for the presence of WNV. Once the testing results came back with a positive mother all her offspring were separated by instar (1^{st} 113, 2^{nd} 143, 3^{rd} 14) and killed via freezing so that they could be tested later for TOT of WNV. All three pools of larvae came back negative for the presence of the virus.

These results should have been expected as literature on TOT suggests many different factors can influence the detection of virus after it has entered the embryo. Some of these factors include an increased egg incubation period before hatching (Mourya et al. 2001), testing offspring from eggs oviposited during the second and third gonotrophic cycles after the initial infectious blood meal (Anderson et al. 2008), and rearing larvae to adults before testing rather than testing the initially hatched larvae (Mourya et al. 2001; Anderson and Main 2006) have all been shown to increase the rate of virus detection. Larvae were not reared to adults because The Public Health Agency of Canada lists WNV as a containment level 3 viruses and as such any live individual suspected of infection must be within a containment Level 3 lab. Rearing the infected females offspring to adults rather than testing them as larvae would have provided a better opportunity to detect WNV TOT. At the time, Brock's CL3 facility had not yet been completed.

As noted, numerous biological and environmental factors may affect TOT and most of these cannot be controlled when conducting field surveillance for TOT. This thesis tested three times the number of individuals that Miller et al. (2000) tested, but they detected TOT in one of their pooled samples suggesting that TOT field surveillance can be associated with sampling in the right place at the right time.

Again, although the results from this study did not discover naturally occurring TOT of WNV, it should not be used as a platform to suggest that this method of virus transmission is not occurring at all or that it is not playing a role in the survival of the virus in Ontario. TOT is a deceptive method of virus survival where the occurrence of a single offspring infected from its mother could facilitate the transmission of the virus for

a long period of time without the need for successful horizontal transmission into a new host.

2.6 Acknowledgements

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Chapter Three

Successful Oral Inoculation of Male and Female Mosquitoes with West Nile Virus using an Infectious Sugar Meal (ISM) Protocol.

3.1 Abstract

A mosquito species' susceptibility to infection and ability to transmit a virus into a new host is considered its vector competency. Three common methods are used to infect mosquitoes with a virus when conducting research in controlled laboratory conditions: (1) intrathoracic inoculation; (2) allowing mosquitoes to feed on live infected vertebrate hosts; or (3) allowing mosquitoes to feed on an infectious blood meal using an *in vitro* blood-feeding protocol.

A different method for infecting mosquitoes is using an infectious sugar meal (ISM). This method was previously shown to result in mosquito infection by researchers in the 1920's, but has not been used in studies investigating vector competency. This alternate infection method has been adapted from the literature and used in a series of trials to determine its suitability for use in studies of West Nile Virus (WNV) infection, dissemination, and transmission in the known vector *Aedes albopictus*. ISM trials resulted in the successful infection of 82 of 123 females and 64 of 113 males tested for a total of 148 of 236 individuals infected; of these, 139 exhibited disseminated infection. The ISM infection rates (82.8%) were significantly higher than those observed in an infectious blood meal fed *in vitro* (37.5%) of the same titer and similar to the observed rates within previous literature (85.5%) using oral infection at a higher titer.

The many benefits associated with using the ISM over the more common infection protocols are discussed. Arguments why this protocol should be considered a viable option when conducting vector competence research are presented.

3.2 Introduction

Simplistic observational studies such as the work conducted by Finlay (1881), provided evidence that deadly human pathogens like Yellow Fever were being transmitted during the blood feeding of mosquitoes. This discovery led to research uncovering different disease transmission capabilities within and among mosquito species. A species' ability to transmit a virus is called its vector competence. For a species to be considered a competent vector, a virus must be capable of infecting and disseminating out of the midgut and into either the salivary glands or sexual organs so that active virions are transmitted horizontally into a new host or vertically into a new vector (Kenny and Brault, 2014). Two main infection methods have been used for inoculation of mosquitoes: oral and mechanical. Both methods have been modified throughout the years by advances in scientific technology.

The most natural infection method route available for conducting transmission research is by orally infecting female mosquitoes. This can be achieved through feeding on a previously infected vertebrate or by feeding on extracted blood mixed with cultured virus. Researchers simply inoculate a vertebrate host with a known virus concentration and wait for a given incubation period. This time, known as the intrinsic incubation period, allows the virus to propagate within the host resulting in a predictable blood titer range with which to infect mosquitoes (Mourya et al. 2001; Turell et al. 2005). In the absence of vertebrate hosts, cell culture can be used to create and quantify stock solutions of a virus (Podlech et al. 2002). These solutions can be mixed with extracted vertebrate blood to create an infectious blood meal within a controlled environment. In laboratory, females have been readily shown to feed on something as simple as a cotton pad soaked

in blood or through more complex membrane and suspension feeders (Rosen 1987; Mourya et al. 2001; Joshi et al. 2002). Several biological signals are triggered prior to initiating the blood feeding behaviour by female mosquitoes, many of which are naturally triggered when live animals are used for infection. Blood meals from non-living hosts in the lab do not activate many of the behavioural cues that entice females to feed resulting in experiments with low sample sizes (Turell et al. 2005; Vaidyanathan et al. 2008). These meals can be manipulated by adding amino acids to extracted blood, using vertebrate skin as a membrane, or maintaining a consistently warm temperature to induce the feeding behaviour (Goddard et al. 2002). Aragão (1929) exploited the sugar feeding behaviour of starved adult mosquitoes to infect females and males with yellow fever virus (YFV). Lothrop et al. (2012) modified this protocol by using West Nile Virus (WNV) (titers from 10^1 to 10^7 PFU/mL) mixed with sugar on cotton pads for infection of *Culex tarsalis* mosquitoes, a species that is ranked similar to *Aedes albopictus* in terms of its WNV vector competency (Table 1.1). Their protocol yielded limited infection (33%) at only the 10^7 titer; no other titers produced infection.

The midgut and salivary gland barriers have both been shown to impede or prevent a virus' dissemination into and out of these important transmission organs resulting in a variable extrinsic incubation period (Anderson et al. 2008; Anderson et al. 2010; Kenney and Brault 2014). Longer incubation periods can limit research into certain aspects of transmission because essential biological processes like digestion and oviposition take time and must occur before a mosquito will complete a second feeding cycle. Survival time has been shown to be limited in infected WNV vectors. Vaidyanathan et al. (2008) showed a 40% mortality rate in *Culex pipiens* 10 days after

infection. It would therefore be beneficial to decrease the extrinsic incubation time as much as possible to increase the likelihood that a sufficient number of infected mosquitoes survive long enough for experiments.

Inoculating virus directly into the haemolymph of the mosquito bypasses the midgut barrier and drastically decreases the extrinsic incubation period (Rosen and Gubler 1974; Dohm et al. 2002; Goddard et al. 2002; Anderson et al. 2008). Rosen and Gubler (1974) were the first to provide a detailed protocol using compressed air attached to a fine tipped needle for repeated injection of infectious solution into the thoracic region of mosquitoes, a process known as intrathoracic inoculation (ITI). The ideal location for ITI is in the neck of males and through the membranous area below the spiracle and anterior to the mesepisternum on the side of a female's thorax. This protocol has emerged as a standard infection method even though it has yet to be determined if the invasive puncturing has any direct consequences on post infection transmission. Han et al. (1999) provide evidence that within 30 minutes of a rupture in the cuticle wall, a mosquito's immune system responds by rapidly producing at least six distinct hemolymph polypeptides. Immune responses come at a cost to the individual as there is an increased physiological and energetic stress when the pathways are activated (Ardia et al. 2012). This additional stress due to the ITI puncture wound could influence several biological pathways, thereby influencing transmission rates.

The first goal of this study was to determine if the protocols for oral infection used by Aragão (1929) and Lothrop et al. (2012) could be modified to infect *Ae. albopictus* mosquitoes by feeding them an infectious sugar meal (ISM). A competent WNV vector refers to the capability for infection, dissemination, and transmission of the

virus by an individual that feeds on an infected host (Goddard et al. 2002). This experiment was also used to assess the efficiency of the ISM by comparing the observed infection, dissemination, and transmission rates with those observed from feeding mosquitoes on an infectious blood meal using an *in vitro* blood-feeding protocol.

Previous literature has shown that the titer of the infectious meal plays a large role in the observed infection and dissemination rates of WNV (Turell et al. 2001; Anderson et al. 2010). Goddard et al. (2002) noted that infection was possible when feeding on a titer as low as 10^3pfu/mL, which was confirmed by the limited infection rate observed in *Ae. albopictus* on a titer of $10^{4.3}$pfu/mL (Akhter et al. 1982). Komar et al. (2003) suggested a threshold titer of 10^5pfu/mL is needed to establish a sufficient transmission cycle. These titer limits were therefore used as a guideline to select the infection titers.

ISM trials were conducted on both sexes with blood infection trials conducted only on females. Each trial was run in triplicate. Two additional ISM trials were run to investigate virus transmission by both sexes. Because the use of live hosts was not permitted in the Containment Level 3 (CL3) laboratory, the ISM trials will be compared to oral infection using a live infected host in previously published studies. If observed rates are similar or higher than those suggested by other authors, then the possibility that the ISM protocol could be beneficial when investigating viral transmission should be considered.

3.3 Methods

3.3.1 Mosquitoes

Ae. albopictus has previously been established as a competent WNV vector as the virus has been detected within wild populations (Gratz 2004) and readily disseminated and transmitted under laboratory conditions (Turell et al. 2001; Sardelis et al. 2002). *Ae. albopictus* colony eggs were purchased from Rutgers University and shipped to Brock University in a humidity controlled container. Eggs from the shipment and subsequent generations were placed into plastic rearing pans filled with dechlorinated tap water that was supplemented with 0.25g brewer's yeast and 0.75g crushed fish food (Omega^{TM} One super color tropical fish flakes) per 1L and maintained at 24°C under 16h:8h (L:D) photoperiod. After hatching, larvae were reared under the same conditions. Approximately 2g of crushed fish food was added every 2-4 days depending on the conditions of the rearing pan. Pupae were collected daily and placed into a mosquito emergence container (BioQuip®, cat. #1425).

Adults were transferred to 30cm x 30cm x 30cm mesh cages and maintained at approximately 24°C, 80%RH, and a 16h:8h (L:D) photoperiod which is standard for laboratory colonies (Vaidyanathan et al. 2008). Colony mosquitoes not used for infection trials were fed 10% sucrose *ad libitum* for daily nutrient support (replaced every 2-3 days, as required). Nutrients for egg production were provided by periodic blood sausages once or twice per week, depending on colony requirements. See Appendix 3 for the blood sausage protocol. Blood sausages fed to colony mosquitoes did not contain virus and therefore, blood-feeding could be conducted outside of a BSC.

Adults used in trial experiments were collected 1-2 days post emergence, sorted by sex in groups of 30, transferred to a housing container (Figure 3.1) and placed under colony conditions until transport to the Containment Level 3 (CL3) laboratory for infection. No sucrose solution was provided to individuals 24 hours prior to infection. After infection, mosquitoes were housed at 25±2°C, 80%RH, and placed under 16h:8h (L:D) photoperiod. Immediately after infection females were provided a 10% sucrose solution *ad libitum* (changed daily).

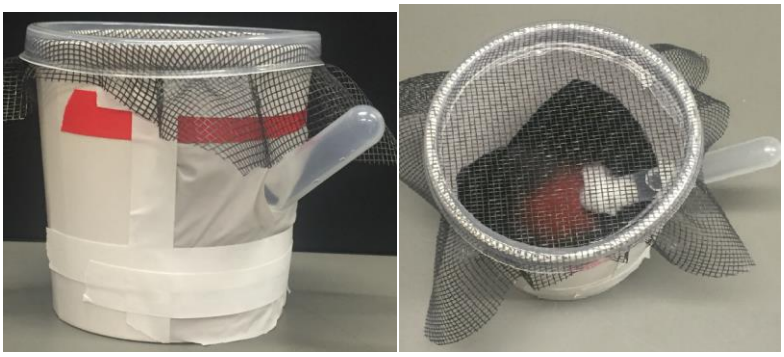


Figure 3.1- Post infection housing for adult mosquitoes. Containers were covered with a fine mesh so individuals could not escape. An entry port was cut into the side of the containers to allow entry and exit of materials. This port was covered inside and out by latex gloves with slits cut in a + shape to inhibit escape. A 5mL conical tube filled with a 10% sucrose solution and a cotton ball for absorption was placed through the side to provide a source of nutrition.

3.3.2 Virus

WNV NY99 strain was obtained from The Public Health Agency of Canada and used for infection. Working stocks of WNV were generated by infecting VERO E6 cells in tissue flasks (Sarstedt AG & Co., Cat. 83.3911.002) with growth areas of 75 cm^2 to 175 cm^2. The supernatant of infected cell cultures was collected and stored in 1mL aliquots at -80°C in the CL3 facility. Stock titers were estimated through the plaque assay equation (Number of plaques/(Dilution Factor x mL of inoculum per plate)) and

determined to be approximately $10^{6.8}$pfu/mL. See Appendix 6 for full virus propagation and plaque assay protocols.

3.3.3 Infected Blood meal Method

An infectious blood meal was provided using a Sausage case Feeding Protocol (as outlined in Appendix 3) using hog intestine casing filled with 2mL stock concentration of WNV NY99 ($10^{6.8}$pfu/mL) and 18mL of citrated sheep's blood (Cedarlane®, Cat. No CL2581-1000C) for a final blood meal titer of approximately $10^{5.8}$pfu/mL.

After feeding, the housing container was transported inside a secondary container (according to CL3 protocol) into a CO_2 knock out chamber for sorting. Mosquitoes were immobilized by exposing them to a low amount of CO_2 so that fully engorged individuals could be sorted under a microscope and placed into a new housing container. Only individuals fully engorged with blood were kept for trials.

3.3.4 Infected Sugar Meal Method

An ISM was provided by feeding individuals a 2mL Dulbecco's Modified Eagle's Medium (DMEM) (Sigma®, Cat. F1015-500ML) solution at a 5% sucrose concentration with either a final low WNV titer of $10^{3.8}$pfu/mL or high WNV titer of $10^{5.8}$pfu/mL. The full ISM feeding protocol can be found in Appendix 3. Mosquitoes were removed from the glass feeding chamber with an aspirator gun and transported according to CL3 protocol into a CO_2 knock out chamber for sorting. Individuals were immobilized by exposing them to a low amount of CO_2 so that fed individuals could be sorted under a microscope and placed into a new housing container. Green food colouring was added to

the ISM to ensure that only fully fed individuals were kept for trials. Only individuals with crops and/or abdomens fully distended with the green ISM were kept for trials.

3.3.5 Dissections

After 10 days post infection, dead mosquitoes were discarded from the housing container and surviving individuals were placed into a -80°C freezer. Individual mosquitoes were dissected and placed into 1.5mL microfuge tubes filled with 1mL of DMEM (Sigma, Cat. F1015-500ML). The legs, wings, and head were taken off using two forceps and placed into a separate tube from the body. Infection was determined by detecting virus within the microfuge tube containing the body and dissemination when the sample containing the removed legs, wings and head tested positive for virus. The dissemination tube was homogenised first and with different forceps than the body. After homogenization, the forceps were immediately disinfected with a series of wash steps using 10% Virkon solution (Vetoquinol, Cat. 0-2353000) and 95% ethanol. A control was created during the dissection of each trial by randomly placing each of the two disinfected forceps into 1mL of DMEM (Sigma-Aldrich, Cat. F1015-500ML) in a 1.5mL microfuge tube (1 tube for each) and swirling the forcep tips in the solution. The control samples were tested for the presence of WNV to ensure no cross contamination occurred between dissection of samples.

3.3.6 Virus Detection

TaqMan® reverse transcriptase polymerase chain reaction (RT-qPCR) was used to test for the presence of WNV RNA in samples. Viral testing was done according to the Public Health Agency of Canada gold standard national guidelines and followed the protocols established by Lanciotti et al. (2000) with some minor changes. Full protocols, including media and reagent recipes for RNA extraction and RT-qPCR can be found in Appendix 4. Dissection and homogenisation were conducted following the protocol previously discussed in section 3.3.4 (not the protocol found in Appendix 4) to distinguish between infection and dissemination.

Samples positive for the presence of viral RNA were further screened *in vitro* to ensure that infectious virions were present within them. Positive sample homogenates were used to inoculate VERO E6 cell culture 6-well plates according to the protocol in Appendix 6. Samples were considered to contain active virus when wells showed signs of cytopathic effect (CE) before control wells (Figure 3.1). A subset of *in vitro* samples showing CE were retested by RT-qPCR to confirm the presence of infectious WNV since inactive RNA would not have penetrated the cells during inoculation and should have been washed out of the well during *in vitro* testing.

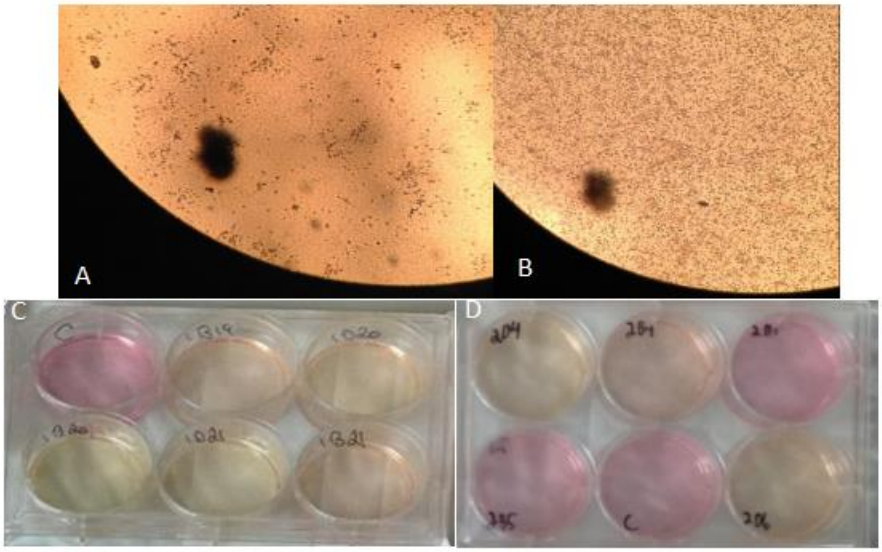


Figure 3.2- VERO E6 cells in 6 well culture plates. A visual difference can be seen between healthy (A) and infected (B) monolayers through the large accumulation of dead cells. DMEM is pH sensitive with acidification indicated through a pink to yellow colour change. The control well (labeled with a C) shows a distinct pink colour as all sample wells have changed towards yellow (C). 2 of the 5 sample wells show no colour or monolayer indication that CE has occurred (D).

3.3.7 Saliva Collection

After 10 days, two replicates of high titer ISM infected females were manipulated to collect and test saliva secretions for transmission of virus using capillary tubes similar to a protocol by Styer et al. (2007) (Figure 3.2). See Appendix 7 for the full saliva collection protocol. All successful saliva collections were tested for viral RNA and screened *in vitro*.

Figure 3.3- Microscopic view of capillary tube saliva collection. Capillary tubes were filled with a sucrose and saline solution. Legs and wings were removed so the female cannot move. Spitting was induced by applying a drop of 10% pilocarpine solution (VWR, Cat. D8537) to the thorax.

3.3.8 Venereal Transmission

After 10 days, three replicates of high titer ISM infected males were placed in a cage with uninfected virgin females to determine if males were capable of transmitting WNV venereally during copulation. The full protocol for venereal transmission can be found in section 4.3.

3.3.9 Statistical Analysis

The infection rate was calculated as the (number of infected mosquitoes)/(total number of mosquitoes tested) and dissemination rate as (number of mosquitoes with a disseminated infection)/(number of infected mosquitoes). The Chi-squared and Fisher exact statistical tests were both conducted at a 95% confidence interval. Chi-squared was used to determine if there was any statistical variance among replicates of the same trial and Fisher exact test to determine variance among trial averages. Rates of WNV vector competency for *Ae. albopictus* from previous literature were also compared to the results of this thesis.

3.4 Results

3.4.1 ISM Infection and Dissemination

In total, 236 mosquitoes (123 females and 113 males) survived 10 days after being fully engorged with ISM and 48 females survived 10 days after being fed on an infected blood meal sausage (Table 3.1). Only samples confirmed by both RT-qPCR and *in vitro* cell culture were counted as WNV positives. Infection was observed in 58/127 and 90/109 individuals at the low and high ISM titers, respectively. Results from the Chi-squared tests showed no significant variance (all p-values >0.05) among the replicate infection rates within each trial; see Appendix 8, Table 3 for all Chi-squared results. Dissemination was observed in 51/58 and 89/90 individuals at the low and high ISM titers, respectively. Chi-squared showed no significant variance (all p-values >0.05) among the replicate dissemination rates within each trial; see Appendix 8, Table 3 for all Chi-squared results.

These results indicate that the ISM protocol was successful for the infection and dissemination of WNV by *Ae. albopictus* mosquitoes. The number of infections between the high ISM and low ISM trials also suggests that the infection protocol produced similar results as would be expected when infecting with different virus titers.

No viral RNA was detected in any of the 30 contamination controls created during dissections. All 90 of the randomly selected subset *in vitro* samples showing CE re-tested as positive by RT-qPCR.

3.4.2 ISM Efficiency

The ISM trials produced significant differences between infection and dissemination rates as denoted by the letters in Table 3.1, these letters were obtained from the Fisher exact p-value results found in Appendix 8, Tables 4 and 5. Both high titer ISM trials showed similar infection rates (p-value = 1) and were both significantly higher than all other ISM trials and the infected blood trial (Appendix 8, Table 4). The low titer ISM trial saw significantly higher infection rates in females compared to males (p-value = 0.032), but neither was significantly different from the infected blood meal trial (p-values= 0.086 and 0.844). In comparison to the studies by Akhter et al. (1982) and Turell et al. (2001), the infection rates of the high titer ISM trials were more similar to the blood infections from Turell et al. (2001), the low titer ISM males were more similar to Akhter et al. (1982), and the low titre ISM females fell almost directly in the middle of the two infection rates from literature.

All ISM trials produced significantly higher dissemination rates than those observed in the infected blood feeding trial (Appendix 8, Table 5). The high titer ISM females showed significantly higher dissemination rates from all other trials (Appendix 8, Table 5) except the high titer ISM males (p-value=0.467). The high titer ISM males and both low titer ISM trials all had similar dissemination rates to each other (Appendix 8, Table 5). All trials produced dissemination rates slightly higher but similar to the dissemination rates observed by Turell et al. (2001). These results suggest that the ISM protocol could be regularly used for WNV infection studies on *Ae. albopictus* as it was more efficient for infection and dissemination as compared to the infectious blood protocol used.

Table 3.1. Comparison of Infection rates and Dissemination rates from different WNV infection methods. Statistical analysis was performed using a Fisher exact test. Columns containing trials denoted with the same letter were not statistically different from one another at a 95% confidence interval. Data indicated by bold text were retrieved from previous studies and were used to compare to the rates from the current study. Avian host data were retrieved from Turell et al. (2001). Membrane data were retrieved from Akhter et al. (1982).

	Infection rate	**Dissemination rate**
ISM $10^{5.8}$pfu/mL Females	48/58 (83%)^{a}	48/48 (100%)^{a}
ISM $10^{5.8}$pfu/mL Males	42/51 (82%)^{a}	41/42 (98%)^{ab}
ISM $10^{3.8}$pfu/mL Females	36/65 (55%)^{b}	32/36 (89%)^{b}
ISM $10^{3.8}$pfu/mL Males	22/62 (35%)^{c}	19/22 (86%)^{b}
$10^{5.8}$pfu/mL Infected blood meal	18/48 (38%)^{bc}	7/18 (39)^{c}
Avian Host $10^{7.2}$pfu/mL	**55/61 (90%)**	**47/55 (85%)**
Membrane $10^{4.3}$pfu/mL	**5/19 (26%)**	**N/A**

3.4.3 ISM Transmission

Results of the venereal transmission trials are discussed in detail within section 4.4 (Table 4.1). A total of 102/225 (45%) virgin females tested positive for viral RNA. Saliva was collected from a total of 15 females from two different infection trials (Table 3.2). Initial RT-qPCR results detected virus in 60% of the total samples tested. CE was observed in all but 1 well of the 15 saliva samples. Not all of the wells showed CE after the same incubation period; some wells showed signs of CE after 12h while others started to show CE at staggered intervals as more incubation days passed. Following propagation, 14/15 saliva samples were positive for the presence of viral RNA.

These results indicate that the ISM protocol can be used to examine transmission of WNV by *Ae. albopictus* as both sexes were able to readily transmit the virus. It also suggests that the ISM protocol can be used as a non-invasive alternative to intrathoracic inoculation of males as no other options exist for infection of the male sex.

Table 3.2- ISM capillary tube saliva collection results. All samples were propagated *in vitro*. RT-qPCR was conducted on all samples before and after propagation.

	Capillary tube positives (before propagation)	***In vitro* positives (after propagation)**
Trial 1	5/7 (71%)	6/7 (86%)
Trial 2	4/8 (50%)	8/8 (100%)
Total	9/15 (60%)	14/15 (93%)

3.5 Discussion

The first goal of this study was to determine if an ISM produces infection, dissemination, and transmission of WNV within the vector *Ae. albopictus*. Infection and dissemination of WNV into the hemolymph was observed within individuals fed both the low and high titer ISM (Table 3.1). Transmission of WNV was shown through the collection of saliva (Table 3.2) and through the testing of virgin females placed in cages with infected males (Table 4.1). The combination of these results indicates that the ISM protocol does produce viable WNV vectors as seen in competence studies using blood infections (Turell et al. 2001).

The second goal of this study was to determine the efficiency of the protocol by comparing the observed infection, dissemination, and transmission rates to those observed through a blood infection and to those observed in the literature (Table 3.1). The infection rates observed from the infected blood meal at a $10^{5.8}$pfu/mL were lower than the blood infection trial at $10^{7.2}$pfu/mL from infected chicks (Turell et al. 2001) and similar to the rates from membrane feeding at $10^{4.3}$pfu/mL titers (Akhter et al. 1982). This was expected as initial infection titer has previously been shown to influence infection rates. A WNV infection study by Tiawsirisup et al. (2004) showed a significant increase in *Ae. albopictus* infection rates of 19%, 50%, 71%, and 87% when feeding on chickens with infectious titers of $10^{6.0}$, $10^{6.5}$, $10^{7.0}$, and $10^{7.5}$ respectively. A similar trend occurred between the high and low ISM trials when compared to each other, but not when they were compared to the infected blood trial.

The $10^{3.8}$pfu/mL low titer ISM trials produced infection rates more similar to the $10^{5.8}$pfu/mL blood infection over the $10^{4.3}$pfu/mL blood infections observed in Akhter et al. (1982). The $10^{5.8}$pfu/mL ISM trials produced rates similar to the $10^{7.2}$pfu/mL blood infection in Turell et al. (2001) as opposed to the infection at $10^{4.3}$pfu/mL in Akhter et al. (1982) or the $10^{5.8}$pfu/mL blood infection trial. A similar deviation from the literature was observed in the dissemination rates; all ISM trials produced results in line with the $10^{7.2}$pfu/mL blood infection in Turell et al. (2001) as opposed to the infected blood trial at $10^{5.8}$pfu/mL of the current study.

These deviations from literature are potentially due to the influence of the infectious meal composition on the midgut infection barrier. Only fully engorged individuals were selected for trials, which suggests that both the blood meal and the ISM

appeared to be diverted to the midgut as only this structure would be capable of such drastic expansion during feeding (Nation, 2008). It has previously been hypothesised that a virus would have an easier time infecting the epithelial cells of the crop as compared to those in the midgut, but under natural conditions there is no evidence that an infectious blood meal would end up in the crop unless the sugar content of the meal was greater than 2.5% (Kenney and Brault 2014). The conditions within the crop would be more inviting for a virus because there are fewer digestive enzymes, there would be no interference from the peritrophic membrane, and there would be more time for infection as the crop maintains the long term storage of carbohydrates (Nation, 2008; Kenny and Brault 2014). Starved male mosquitoes have previously been shown to rapidly digest their entire first sugar meal after emergence while starved females will store at least some volume for future use (Clements, 1992). The different rates of sugar digestion would explain why increased rates of infection and dissemination were observed in the female over the male ISM trials as females would retain at least some of the ISM in the crop for long term energy requirements as opposed to rapid digestion of the entire meal. More time without digestion and the following excretion of waste could be the key difference in the observed infection rates between the sexes and would also explain why the ISM trials were more similar to infected blood meal infections at a higher virus titer.

Increased ISM infection rates were not observed in the sugar infection trials by Lothrop et al. (2012). Their results yielded no infection from an ISM below their high titer of 10^7pfu/mL and only observed a 33% infection rate in *Cx. tarsalis,* a WNV vector that has been shown to be comparable to *Ae. albopictus* (Turell et al. 2005). A key difference between Lothrop et al. (2012) and the current study was the method that the

ISM was delivered to the mosquitoes. Lothrop et al. (2012) dipped a cotton pad into the ISM and left it for the mosquitoes to feed on for several days. They noted that after 48h each of their ISM showed a significant increase in RT-qPCR C_T values (less viral RNA detected) and was not detectable from *in vitro* cell culture (no infectious virus remained). There is potential that some of the individuals tested in their trials did not feed on the solution before its infectivity had degraded as there was no indication that mosquitoes had fully engorged on the ISM within a given time. The current protocol allowed a 30min window for feeding and discarded any individuals not fully engorged, possibly explaining the higher rates of infection and dissemination observed.

Saliva collections from 15 females were tested via RT-qPCR initially showed a successful transmission rate of 60% that jumped to 93% (5 additional positive samples) after *in vitro* inoculation on VERO E6 cells. This indicates that there was active virus within the 5 additional saliva collections not initially detected by RT-qPCR. Styer et al. (2007) suggest that saliva containing virus titers as low as 10^2pfu/mL can be expelled during feeding *in vitro*. This might explain why detection only occurred after propagation because the initial saliva collections were diluted in 500mL of DMEM which could have diluted the viral titer below detectable levels (Lanciotti et al. 2000). VERO E6 cells in culture have no defense against an active virus and infection of one cell with a single virion should result in the exponential amplification of the virus (Desmyter et al. 1968; White 1987; Payne et al. 2006). Turell et al. (2001) estimated the transmission rate of their orally infected *Ae. albopictus* to be approximately 73% which is similar to the 60% transmission rate detected from the initial saliva collections tested by RT-qPCR.

Because the ISM trials produced similar vector competence results to those from the literature, several benefits in using this protocol should also be discussed. The ingredients for the ISM are cheap to purchase and last longer than extracted blood. The protocol does not require labour associated with the upkeep of live vertebrate animals in the lab. Furthermore, it eliminates some of the difficulties associated with feeding wild caught mosquitoes within the lab as initiating sugar feeding behaviour is much less complicated than initiating blood feeding which requires several behavioural cues to be triggered (Kenney and Brault 2014). This difference should allow for a larger number of wild caught mosquitoes to feed on the ISM solution as opposed to feeding on infected blood through an artificial membrane. The current protocol resulted in the testing of 123 females that fed on the ISM and survived the extrinsic incubation period. These numbers are much higher than the 48 females that fed on the infected blood in the current study as well as being higher than 19 females in Akhter et al. (1982) and 61 females in Turell et al. (2001). The ISM also provides advantages for downstream transmission studies as disseminated infection can be produced without the development of eggs. Anderson et al. (2008) and Mourya et al. (2001) both showed evidence that transovarial transmission rates of WNV and Dengue Virus (DENV) increased in subsequent gonotrophic cycles following the infectious meal. The ISM would result in disseminated virus into the reproductive tract of females prior to the production of eggs from a blood meal exactly like infected females producing their second batch of eggs would experience.

As the global spread of mosquito-borne viruses reaches new locations the need to understand which local species pose the greatest risk for transmission of a virus is extremely high. The observed rates of infection, dissemination, and transmission when

using the ISM protocol produced similar results to those of Turell et al. (2001) when assessing the WNV vector competency of *Ae. albopictus*. It is the only method to infect male mosquitoes with WNV aside from the invasive intrathoracic inoculation technique and it provides the ability to readily infect a large number of wild caught individuals within the laboratory setting. When considering the evidence provided throughout this study, the use of the ISM should therefore be considered as an alternative method when assessing vector competency of WNV.

3.6 Acknowledgements

This study was supported by an NSERC Discovery Grant to Dr. F.F. Hunter. We would like to thank Bryan, Darrell, and Mariana for the countless hours spent within the CL3 facility, Larissa for her amazing work setting up and maintaining the lab equipment, the Brock University facility management staff for all their work getting the CL3 lab running, and the Public Health Agency of Canada for their guidance and support during the beginning stages of our lab's vector research.

Chapter Four

Male to female venereal transmission of the West Nile Virus by artificially infected *Aedes albopictus* mosquitoes.

4.1 Abstract

Female *Aedes albopictus* are a proven vector of West Nile Virus (WNV) and have been shown to vertically transmit the virus to their offspring. Progeny that emerge as infected adult females can transmit the virus to uninfected hosts while taking the blood meal that is required for egg development. Recent evidence suggests that venereal transmission (VT) from vertically infected males into uninfected females occurs as infectious virus is transmitted in the seminal fluid during mating. This process has been identified in several epidemiologically important viruses, including WNV.

A novel method for orally infecting mosquitoes with an infectious sugar meal (ISM) resulted in the infection, dissemination, and transmission of WNV within female *Ae. albopictus*. The same protocol was also shown to result in the infection and dissemination of WNV within males of the same species. The current study was conducted to determine if male *Ae. albopictus* could VT WNV to uninfected virgin females during copulation.

Viral RNA was detected in 45% of post-mating females following copulation with infected males, providing evidence that the ISM resulted in the VT of WNV from males to females. A thorough review of literature also indicates that this is the first recorded evidence that the invasive WNV vector *Ae. albopictus* is capable of venereally transmitting WNV from an infected male to an uninfected female.

4.2 Introduction

There are several ways in which a virus can successfully infect a new mosquito vector without the requirement of blood feeding. Vertical transmission is the direct transmission of a pathogen with no intermediate host. The most common route of vertical infection in mosquitoes is through transovarial or transovum transmission (TOT) where an infected mother passes the virus to her offspring. Numerous flaviviruses have demonstrated TOT in laboratory experiments, including West Nile Virus (WNV) (Baqar et al. 1993; Dohm et al. 2002). Baqar et al. (1993) and Dohm et al. (2002), infected five different species of mosquitoes (*Ae. albopictus, Ae. aegypti, Cx. pipiens Oc. triseriatus* and *Cx. tritaeniorhvnchus*) with WNV through intrathoracic inoculation (ITI) and found evidence of WNV TOT in each. Detection of virus within wild caught adult males provides natural evidence of TOT as the sex is highly unlikely to have acquired infection from any source other than their infected mother (Collins 1963; Thompson and Beaty 1978; Mourya et al. 2001). Miller et al. (2000) were the first to discover WNV from male mosquitoes and others since have discovered infected wild males (Andreadis et al. 2001; Goddard et al. 2002; Unlu et al. 2010) and infected male adults reared from wild collected eggs and larval stages (Reisen et al. 2006). Unlu et al. (2010) reared wild collected *Ae. albopictus* larvae in the laboratory and detected WNV RNA in adult females providing evidence that the vector species was capable of vertically transmitting the virus to its offspring.

Venereal transmission (VT) is a mode of vertical transmission where a pathogen carried in the seminal fluid of vertically infected males enters females during mating resulting in their infection (Clements, 2011). The few studies conducted on VT has

provided evidence that Dengue virus (DENV) (Rosen, 1987), St. Louis encephalitis virus (SLEV) (Shroyer, 1990), Japanese encephalitis virus (JEV) (Rosen et al. 1989), Chikungunya virus (CHKV) (Mavale et al. 2010), and WNV (Reisen et al. 2006) are all capable of being transmitted from male to female during copulation. Rosen (1987) infected male *Ae. albopictus* with DENV 1-4 (using ITI) and introduced them to uninfected virgin females either 7 or 14 days later. In total, 158 females mated with 7 day post infection males and 135 with 14 day post infection males. The study observed VT rates ranging from 0-12% in 7 day trials and 15-64% in the 14 day trials. The study by Reisen et al. (2011) tested the WNV VT rate within *Culex tarsalis.* They successfully force mated 21 females with infected males and held 3 overnight and 18 for 3-5 days. Viral RNA was detected in 2/3 and 1/18 females, with 1 of them containing enough infectious particles to be identified in plaque assay.

The polygamous behaviour of mosquitoes is a key component to the potential impact of infected males in nature. Choochote et al. (1998) observed a single female *Ae. albopictus* placed in a cage with 10 males could successfully mate with 3 different males in a single hour. Polygamy was also shown in males when Boyer et al. (2011) observed a single male placed among 20 females mated with an average of 9.5 and a maximum of 14 over 7 days. A sustained infection within a single male mosquito could transmit virus into upwards of 10 females within a single week.

The current study was conducted to determine if *Ae. albopictus* males infected using an infectious sugar meal (ISM) could transmit WNV venereally to uninfected virgin mosquitoes during mating. The goal was to place a group of uninfected virgin females into a cage with males that had been infected via ISM 10 days earlier and to

determine whether VT had occurred by testing the mated females for WNV by qRT-PCR after 7 days.

4.3 Methods

The current study was conducted using the same protocols found in chapter 3 with minor changes listed below.

4.3.1 Mosquitoes

Ae. albopictus has previously been established as a competent WNV vector and males have been shown capable of transmitting DENV to females (Rosen 1987; Turell et al. 2001; Sardelis et al. 2002). Full protocols for maintenance and selection of adults can be found in section 3.3.1 with the only minor change being that adults selected for VT infection were separated into three trials of 50 males each. Newly emerged females were collected from emergence containers 8 or 9 days after the infection of males and placed into colony cages to ensure they remained virgins. Three groups of 75 virgin females were moved into housing containers on day 10 and brought into the containment level 3 (CL3) laboratory to be mated with infected males.

4.3.2 Viral Infection

Full protocols for virus selection and male infection can be found in sections 3.3.2 and 3.2.4 with the following minor changes. Males were infected by feeding on 2mL of a high titer 10^5pfu/mL infectious sugar meal ISM. To ensure each of the three ISM were created equally, a single 6mL ISM was divided into three 2mL solutions, one for each trial.

4.3.3 Venereal Transmission

At 10 days post infection, 5 random males from each trial were freeze-killed to confirm the infection rate of males within each trial. 25 males were taken out of each housing container and placed in larger colony cages (30cm x 30 cm x 30cm, to increase available flight room for mating) with 75 uninfected virgin females. A 10% sucrose solution, changed daily, was provided *ad libitum* to each cage. Cages were maintained at the same conditions as the housing containers. After a 7 day mating period all adults were freeze-killed in cages and the 25 males from each trial were separated from females under a dissection microscope and stored at -80°C until viral testing.

The presence of viral RNA from a virgin female was considered positive for VT as previous literature has ruled out the possibility that females could became infected from feeding on the same sugar source as infected males (Mavale et al. 2010) or other infected females (Lothrop et al. 2012).

4.3.4 Dissections

The 5 males from each trial that had been killed on day 10 were dissected using the same protocols found in section 3.3.5 except that only 1 contamination control tube was created for each trial. The remaining males and females were not dissected for dissemination prior to being placed into 1.5mL microfuge tubes. The entire mosquito was placed into a 1.5mL microfuge tube with 1mL of DMEM and immediately homogenized using forceps. All other dissection steps were followed using the protocol found in section 3.3.5 including the creation of the contamination controls.

4.3.5 Virus Detection

TaqMan® reverse transcriptase polymerase chain reaction (RT-qPCR) was used to determine the presence of WNV RNA and only the males collected on day 10 were tested for infectious virus using *in vitro* cell culture. The protocol described in section 3.3.6 was followed for both detection methods. WNV has previously been shown to become inactivated (non-infectious) within infected females that died two days prior to testing in cell culture (Turell et al. 2002). However, RT-qPCR detected WNV RNA from the same infected females held at room temperature up to 20 days after death (Turell et al. 2002). Due to these results, the VT males that were collected after mating were not tested using *in vitro* cell culture because any males that might have died during the 7 day mating period were not removed from the cage.

4.3.6 Statistical Analysis

A Fisher exact test at a 95% confidence interval was used to determine if there were VT rate differences between the three trials.

4.4 Results

The males collected 10 days after infection were used to represent the initial infection and dissemination rate among the remaining males used in each VT trial. In each of the three trials combined, 12/15 individuals showed infection with 11/12 positive for dissemination of WNV (Table 4.1). The remaining males were collected from mating cages after 7 days with viral RNA detected in 21, 19, and 22 males from trial 1, trial 2, and trial 3, respectively, for a total of 83% (Table 4.1). Viral RNA was detected in 38, 31,

and 33 of the 75 females from trial 1, trial 2, and trial 3, respectively for a total VT rate of 45% (Table 4.1). Viral RNA was not identified in any of the 15 contamination control samples. No significant difference was determined by the Fisher exact test between the 3 female VT trials as all 3 p-values were > 0.3; Trial 1 vs. 2- 0.326, Trial 1 vs. 3- 0.513, and Trial 2 vs. 3- 0.745.

Table 4.1- Results of testing the males and females used during the VT trials. The males at day 10 were tested using RT-qPCR and confirmed infectious through *in vitro* cell culture. The males and females collected 7 days after mating were tested only by RT-qPCR.

		Trial 1	**Trial 2**	**Trial 3**	**Total**
Day 10 males	**Infected**	4/5 (80%)	4/5 (80%)	4/5 (80%)	12/15 (80%)
	Disseminated	4/4 (100%)	3/4 (75%)	4/4 (100%)	11/12 (92%)
VT Males	**Infected**	21/25 (84%)	19/25 (78%)	22/25 (88%)	62/75 (83%)
VT Females	**Infected**	38/75 (51%)	31/75 (41%)	33/75 (44%)	102/255 (45%)

4.5 Discussion

The field collection of adult males infected with a *Flavivirus* provided evidence that males naturally become infected through vertical transmission. Preliminary research on this phenomenon indicated that virus was unable to transfer from infected females to males during mating, suggesting the only method for male infection was from virus entering the egg while inside its infected mother (Rosen, 1987; Jiang et al. 2006). From an evolutionary standpoint, genetic traits should become established in a viral genome if they aid in the vertical transmission of a virus resulting in its survival when horizontal

transmission conditions are not favourable (genetic bottle neck). Transovarial transmission of virus into a male only facilitates survival if the virus is transmitted out of the male and into a new vector or host. VT accomplishes this and it has previously been demonstrated for WNV by *Cx. tarsalis* (Reisen et al. 2006) and by *Ae. albopictus* males with DENV (Rosen, 1987). This indicates that the observed results are the first recorded for WNV transmission by male *Ae. albopictus*.

The VT of DENV 1-4 serotypes by *Ae. albopictus* were shown to occur at rates of 64%, 17%, 16%, and 15% respectively when virgin females were introduced to infected males following intrathoracic inoculation 14 days earlier (Rosen, 1987). The ISM VT rates were quite similar to their DENV-1 trials but slightly higher than the other 3 serotypes. It was expected that DENV-1 and WNV would show more similar VT rates than DENV 2-4 in *Ae. albopictus* because both have been shown to infect and be transmitted at higher rates within North American populations (Mitchell 1991; Turell et al. 2001). Because the two viruses had previously shown similar rates of infection and dissemination it makes sense that they would also show similar rates of VT as opposed to DENV 2-4 serotypes which were not similar in their ability to infect throughout the vector species.

WNV VT has been shown to occur under laboratory conditions (Reisen et al. 2006), but at rates more comparable to the DENV 2-4 serotypes (Rosen, 1987). Reisen et al. (2006) force mated infected *Cx. tarsalis* males with uninfected virgin females and detected WNV in 2/3 females (tested next day) and 1/18 females (tested 3-5 days after mating) for a total VT rate of 14% (3/21). Aside from the different WNV vector species used, the lower VT rates compared to the current VT trial could be due to differences in

the protocols used. Reisen et al. (2006) force mated males after a 7 day extrinsic incubation period with no additional days provided for mating. The ISM VT trial males were given 10 days for extrinsic incubation and an additional 7 days for mating, which could have resulted in a higher viral titre within the male accessary glands before mating. Rosen (1987) showed the influence of extrinsic incubation with males infected 14 days rather than 7 days earlier showed VT rates increase by 50%, 15%, 16%, and 12% for DENV serotypes 1-4, respectively.

The polygamy of mosquitoes also could have played a role in the differences observed between the two studies as force mating only allows a single transmission opportunity. Caged female *Ae. albopictus* are capable of mating with 3/10 males in a single hour and males can mate with up to 4 females a day with an average of 9 to 10 females inseminated over 7 days (Choochote et al. 1998; Boyer et al. 2011). Insemination by multiple infected males would theoretically result in more viral particles being transmitted into females which, in turn, would explain the increased rates of VT observed in experiments that allow for polygamous behaviour.

The recent range expansion of *Ae. albopictus* suggests that the temperate climate of North America is no longer hindering the establishment of populations towards the northern border of the United States. The unfavourable conditions of winter are readily overcome by the species as they can stay dormant for long periods protected from freezing or desiccation in a state of diapause (Sota and Mogi 1992), which has also been shown to increase rates of vertical transmission (Mourya et al. 2001). The ability of *Ae. albopictus* to transmit WNV venereally from males to females adds to a growing list of evidence that the importance of vertical transmission is being underestimated. It also

brings into question the rate at which other local species can VT the virus among their populations.

This information can be used to encourage public health officials involved in WNV surveillance programs to make simple adjustments like including males in the program to account for this type of virus activity. The successful transmission of the WNV by males also provides further evidence that the ISM protocol can be used as a sufficient substitute for WNV infection studies. It would also highlight the potential impact that vertical transmission cycles might have on virus survival and suggest that current protocols for WNV surveillance could be missing potential positives within their programs by not including the screening of males.

4.6 Acknowledgements

This study was supported by an NSERC Discovery Grant to Dr. F.F. Hunter. We would like to thank Bryan, Darrell, and Mariana for the countless hours spent within the CL3 facility, Larissa for her amazing work setting up and maintaining the lab equipment, the Brock University facility management staff for all their work getting the CL3 lab running, and the Public Health Agency of Canada for their guidance and support during the beginning stages of our labs vector research.

Chapter Five

Overall Discussion

The specific objectives of this thesis were to investigate vertical transmission of WNV: 1) under natural conditions using evidence from recent literature to determine if this type of transmission cycle was occurring in Ontario; and 2) by conducting infection studies on live vectors to estimate the rate at which it is occurring. By achieving these objectives, this thesis could provide important information used to modify current WNV surveillance protocols.

The second chapter of this thesis took into consideration many of the pitfalls faced by studies aimed at estimating the naturally occurring rate of TOT. The study considered the high likelihood that an egg, larvae, or male collected in the field was not from an infected female mother. Rather then wasting time and resources testing randomly collected individuals, gravid females were collected in the field and only those that provided eggs in captivity were tested for the presence of WNV. A high priority was placed on collecting *Cx. pipiens* as opposed to other vector species because it was previously identified as the main WNV vector in Canada because it feeds preferentially on the WNV avian host reservoir (Kramer et al. 2008). It has always been hypothesised that TOT was a method for a virus to survive long periods in conditions that did not promote the blood feeding behaviour of the mosquito vector (Aitken et al. 1979; Dohm et al. 2002). Mulyanto et al. (2012) provide evidence contrary to this hypothesis as they observed increased rates of DENV TOT in field collections during prolonged rainy seasons as opposed to prolonged drought seasons. Lengthy periods of rain would increase the vector population resulting in the opportunity of more adult blood meals and a peak in virus transmission. Males were therefore selected from field collection traps set up during the second highest year of virus activity in Ontario, the 2012 field season.

Despite efforts to increase the likelihood of detecting WNV TOT and 3 times the volume of samples tested by Miller et al. (2000) (1000 individuals in 152 pools vs 301 in 56 pools), none tested positive for the virus. The lack of TOT detection was expected as only 1 of the gravid females tested positive for WNV. The offspring from the gravid female were the only samples guaranteed to come from an infected mother. This highlights the downfall of the experiment as a much larger sample of positive gravid females would be needed to better represent the TOT rates in nature. It was also unfortunate that none of the male mosquitoes tested positive as WNV was very active during the 2012 field season. Surveillance for TOT is very much associated with the serendipity of collecting at the "right place at the right time". The results from this chapter highlight many of the difficulties associated with natural detection of TOT and put emphasis on the importance for laboratory investigations into the different processes altering the rate of TOT.

The original goal within the CL3 laboratory was to investigate aspects of WNV TOT by *Ae. albopictus*. These initial experiments were designed with the assumption that females would readily feed on infectious blood meals (Akhter et al. 1982; Vaidyanathan et al. 2008) within the lab. Preliminary trials showed very little feeding success with at most 4 of 50 females engorged after the initial feeding period. While trying to increase the successful feeding rate, an alternative infectious solution was created to test if a group of individuals would orally ingest the mixture at a higher rate than what was being observed with artificially infected blood. The ISM was created by modifying the protocols found in Aragão (1929) and Lothrop et al. (2012).

The third chapter of this thesis was designed to determine if this novel method could be used as a substitute for infecting individuals and determining their vector competency for a virus. To accomplish this goal, ISM experiments were compared with artificial infection experiments and to previous literature in which the WNV vector competency of *Ae. albopictus* was investigated. The results from the study indicated that infection and dissemination were observed at both ISM titers producing similar results among the replicates within each trial.

The observed ISM infection rates were unexpected as infection and dissemination from ISM trials produced results more comparable to blood infections with higher titers. It was initially thought that the low titer ISM would not produce detectable infection as this level of viral titer was suggested to be below the necessary threshold for a sustained transmission cycle (Goddard et al. 2002; Komar et al. 2003). Tiawsirisup et al. (2004) provide a good representation of the influence the infectious blood meal titer has on WNV infection in *Ae. albopictus* as rates of 19%, 50%, 71%, and 87% were observed when feeding on chickens with infectious titers of $10^{6.0}$, $10^{6.5}$, $10^{7.0}$, and $10^{7.5}$pfu/mL respectively. Each half jump in titer magnitude resulted in a significant increase in the trial's infection rate, a trend that is confirmed in similar infection studies (Akhter et al. 1998; Turell et al. 2001; and Anderson et al. 2010). The low titer ISM of $10^{3.8}$ produced results more closely associated with blood meal titers of $10^{5.8\text{-}6.5}$pfu/mL and the high titer of $10^{5.8}$pfu/mL produced results similar to *in vivo* blood meal titers of $10^{7.2\text{-}7.5}$pfu/mL. It was previously hypothesised that diversion of an infectious blood meal into the crop could result in higher infection due to the different conditions within this section of the digestive tract (Kenney and Brault 2014).

The long-term energy storage exhibited by females explains why a large dissemination rate gap was observed between sexes in the low titer trials. If the initial sucrose meal is sufficient, females will reserve a portion for future energy requirements by diverting it to the crop and slowly digesting it over time, as opposed to males who readily digested sugars and extracted the entire source of energy obtained from their first sucrose meal (Nation 2008; Clements 1992). If indeed the sugar content of the infectious meal alters susceptibility of the vector, it would be interesting to identify if a threshold level exists for starved females. This could be achieved by gradually lowering the sucrose concentrations of ISMs and raising the sugar concentration of artificial blood meals to determine if there is any association with infection rates and sugar content of ISM.

Transmission was observed initially in 60% of females and 93.3% after *in vitro* propagation. Even if only the initial detection of viral RNA in saliva samples is considered, the observed rate of 60% (5/9) is comparable to the 86% (12/14) transmission rate observed by Turell et al. (2001). This study was therefore able to provide evidence that WNV infection with the high titer ISM produced similar infection, dissemination, and transmission rates as those observed in *Ae. albopictus* when orally infected with a blood meal titer of approximately 10^7pfu/mL.

These results indicate that the ISM method of infection could serve as a viable option for testing the vector competency of other flaviviruses as well. This could be used as a powerful tool for testing the ZIKV vector status of local mosquito populations as it was recently been declared a public health emergency of international concern (WHO, 2016). The difficulties of enticing wild mosquito populations to ingest blood at a sufficient rate using *in vitro* blood-feeding protocols directly impacted this thesis and

have been well documented to limit many laboratory infection studies. The blood feeding behaviour of females is complex, involving a number of signals and cues such as temperature, carbon dioxide, olfactory stimuli, and visual stimuli (Kenney and Brault 2014). By conducting vector competence studies using ISM, researchers would be able to easily feed a maximum number of first generation wild caught females quickly producing results to determine the vector status of a species.

The fourth chapter specifically highlighted a benefit of ISM inoculation as the VT of WNV from male to uninfected female *Ae. albopictus* was tested. The only other methods for infection of males with WNV is through ITI or TOT from an infected mother. The simple production of the ISM overcame the lack of required equipment for the safe ITI of male mosquitoes. The viral RNA detected from 45% of females mated to infected males is the first reported occurrence of WNV VT by *Ae. albopictus*. The observed infection rate was similar to the rates observed by the vector species for DENV-1 (Rosen, 1987), but higher than the 14% VT rate observed in *Cx. tarsalis* (Reisen, 2006). There were 3-10 additional extrinsic incubation days given to males in the ISM VT studies as compared to the study by Reisen et al. (2006) in which they force mated males after 7 days. Force mating also removed the potential infection rate increase due to the polygamous behaviour of both sexes (Choochote et al. 1998; Boyer et al. 2011). Seven additional extrinsic incubation days resulted in a large jump in the VT of DENV-1 (Rosen, 1987). It would be interesting to determine how both extrinsic incubation time of males and the polygamous behaviour of the insects influences the VT rates. A future study could force and cage mate infected males at the same 7 day interval of Rosen (1987) to identify the influence of extrinsic incubation and polygamous mating.

The finding that the invasive *Ae. albopictus* vector can VT WNV adds to a growing list of literature that highlights the underestimation of the role vertical transmission plays in the survival of a virus (Martins et al. 2012; Mourya et al. 2001). These results are also important when considering the vector's recent range expansion. The temperate climate of North American is no longer hindering *Ae. albopictus* as new populations are expanding northwards. The role that this species will play in WNV transmission within these newly established locations is yet to be determined. Its observed VT rates promotes the need to identify the VT rate in other WNV vector species such as the main north American vector *Cx pipiens* and the most abundant vector species in Ontario *Ae. vexans*.

This thesis highlighted the difficulty in field detection of WNV TOT. Several approaches were used to increase the detection probability, but still no natural cycle was identified within the tested samples. The lack of detection should not be used as a platform against the suggested rate of TOT in nature and instead encourage a larger scale field study for better detection of this elusive transmission cycle. The VT abilities of male *Ae. albopictus* provides more evidence to this point as these secondary transmission cycles are readily demonstrated in the lab. In addition to *Ae. albopictus*, it would be beneficial to identify WNV VT in other important vector species since further positive results might suggest that males be included in surveillance programs. No additional changes to the current program would be required aside from the inclusion of males into a sample pool rather then casting them aside. Having a better understanding on all aspects of virus transmission is an essential part of preparing an efficient surveillance program, especially considering the current state of global epidemics. Identifying which local

species are viable vectors of currently expanding viruses like CHKV and ZIKV is more important than ever. The efficiency, precision, and comparable vector competence results indicate that the ISM is a worthwhile endeavour for identifying potential threats from expanding human pathogens.

Appendix 1- North American Vector Species of West Nile virus

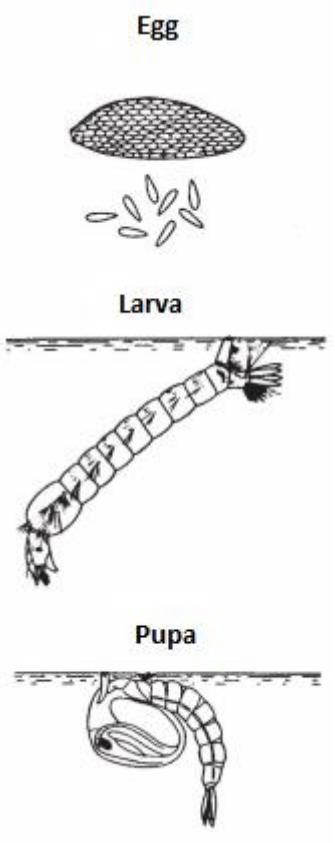
Table 1- Mosquito species in which West Nile virus has been detected, United States, 1999-2012. Retrieved from Centers for Disease Control and Prevention (2012)
* This species was detected in 2003 in Monroe County, FL; but was not reported to ArboNET

Aedes aegypti
Aedes albopictus
Aedes atlanticus/tormentor
Aedes atropalpus
Aedes canadensis
Aedes cantator
Aedes cinereus
Aedes condolescens*
Aedes dorsalis
Aedes dupreei
Aedes epactius
Aedes fitchii
Aedes fulvus pallens
Aedes grossbecki
Aedes infirmatus
Aedes japonicus
Aedes melanimon
Aedes nigromaculis
Aedes provocans
Aedes sollicitans
Aedes squamiger
Aedes sticticus
Aedes stimulans
Aedes taeniorhynchus
Aedes triseriatus
Aedes trivittatus
Aedes vexans
Anopheles atropos
Anopheles barberi
Anopheles bradleyi/crucians
Anopheles franciscanus
Anopheles freeborni
Anopheles hermsi
Anopheles punctipennis
Anopheles quadrimaculatus
Anopheles walkeri
Coquillettidia perturbans
Culex apicalis
Culex bahamensis
Culex coronator
Culex erraticus
Culex erythrothorax
Culex nigripalpus
Culex pipiens
Culex quinquefasciatus
Culex restuans
Culex salinarius
Culex stigmatosoma
Culex tarsalis
Culex territans
Culex thriambus
Culiseta incidens
Culiseta impatiens
Culiseta inornata

Culiseta melanura
Culiseta morsitans
Culiseta particeps
Deinocerites cancer
Mansonia titillans
Orthopodomyia signifera
Psorophora ciliata
Psorophora columbiae
Psorophora ferox
Psorophora howardii
Uranotaenia sapphirina

Appendix 2- Lifecycle and Anatomy of an *Aedes* Mosquito

2.1 Larval stage



Larvae

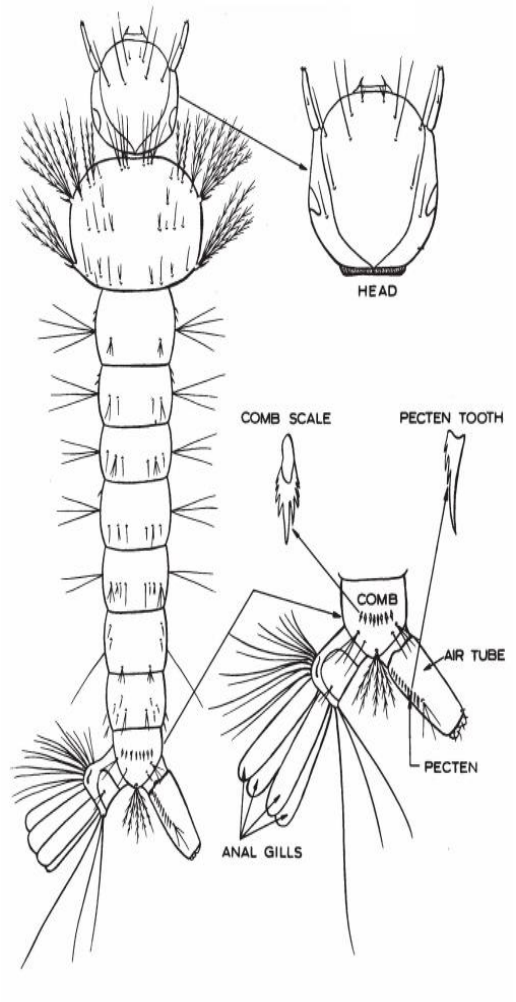


Figure 1. Late instar larval morphology of *Aedes* mosquito (retrieved from Wood et al. (1979)).

2.2 Male Anatomy

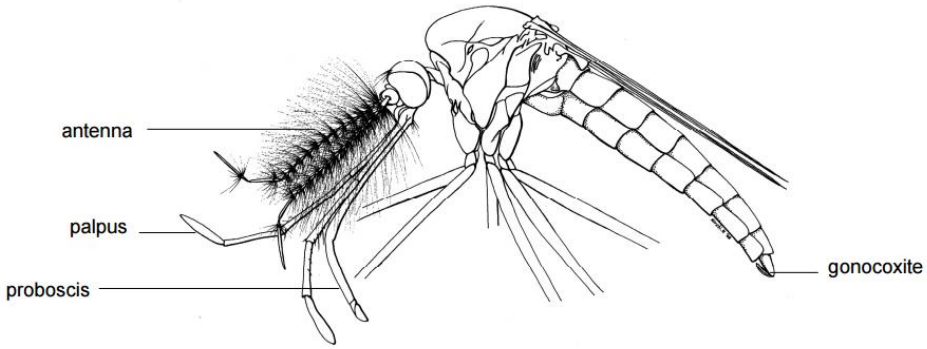


Figure 2. Male *Aedes* anatomy. Males are easily distinguished from females by the plumose antenna, hairier abdomen, and generally are smaller in size than females (retrieved from Thielman and Hunter (2007)).

2.3 Female Mosquito Anatomy:

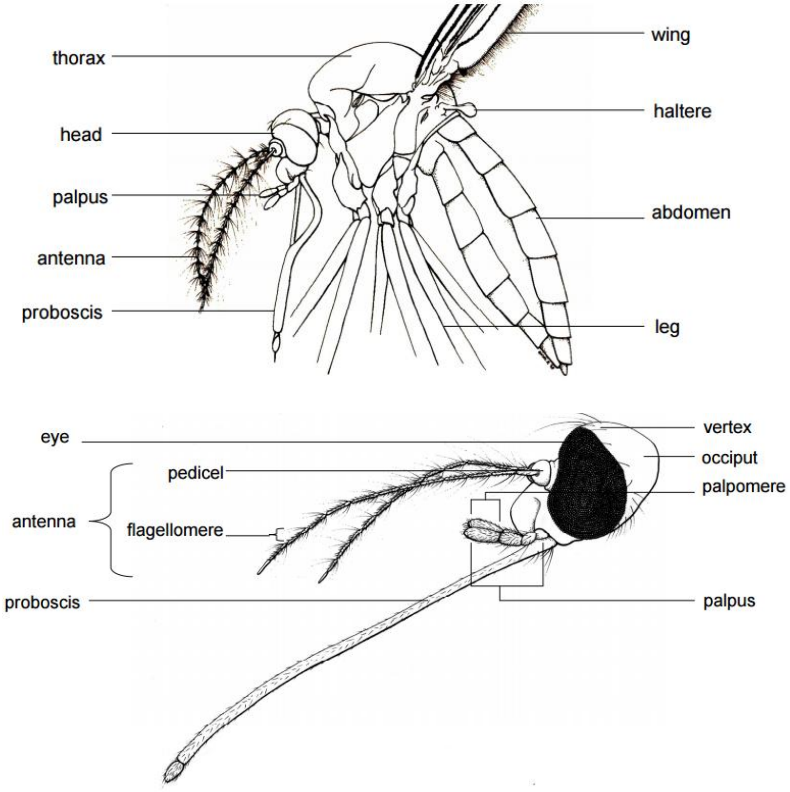


Figure 3. Female *Aedes* anatomy. Females are easily distinguished from males through their generally larger size, having much less seta on the abdomen, and a noticeable lack of a plumose antenna, and their reduced palpi (retrieved from Thielman and Hunter (2007)).

Appendix 3- Oral Infection Protocols

3.1 Blood Sausage

1. Place housing container with mosquitoes to be blood-fed inside of BSC.
2. Cut a length of sausage casing and rinse thoroughly in warm tap water. Soak until ready for use.
3. The blood meal is mixed in a 15mL conical tube and includes:
    a. 18 mL of citrated sheep's blood (Cedarlane Labs, cat.# CL2581-100D) supplemented with 2 mM adenosine triphosphate (ATP)
    b. 2 mL of stock virus ($10^{6.8}$pfu/mL) thawed to room temperature
4. Mix blood meal thoroughly with 10ml pipette
5. Tie a knot at one end of the sausage casing.
6. Use a funnel to fill the casing with the infected blood solution.
7. Tie a knot at the other end. Place sausage into a 500mL glass bottle and seal the lid with parafilm. Place bottle into the water bath at 35-38°C for 30 – 45min.
8. Place blood-filled sausage on the mesh top of the mosquito housing container, cover with aluminum foil, close the sash on the BSC, and turn off the BSC fans and lights.
9. Allow 30 min for feeding.
10. Dispose of blood-filled sausage into a biohazard bag. Wipe off any blood that may have spilled onto the top of the mosquito housing container. Close the BSC and wait 30 min before cleaning.
11. Use an aspirator gun inside of the BSC to remove all females. Surface decontaminate the aspirator collection chamber tip and transport the females to the CL3 knockout chamber.
12. Separate engorged females and immediately place the specimens into a new housing container not used for oral infection trial.
13. Provide mosquitoes *ad libitum* with 10% sucrose and incubate as desired.

3.2 Infectious Sugar Meal (ISM)

1. Place the housing container filled with colony mosquitoes inside the BSC.
2. Ensure that all required instruments and solutions are prepared and positioned at the proper locations before starting.
3. Take the required volume of stock virus from the -80°C freezer, transport to BSC in a secondary container and cover from light inside the BSC.
4. Use an aspirator gun to move mosquitoes from the housing container and place them into a glass feeding chamber (Figure 1).
5. Combine the ISM ingredients into a 15mL conical tube:
    a. 0.2mL of stock concentration ($10^{4.8}$pfu/mL or $10^{6.8}$pfu/mL)
    b. 1.549mL of DMEM
    c. 0.25mL of a 40% sucrose solution
    d. 0.001mL of green food colouring
6. Warm ISM in water bath to 33°C.
7. Slowly and carefully pipette warmed ISM into the circular groove created by the convex center of the glass feeding chamber (Figure 1). Take care that the solution only fills the circular groove and does not cover the raised center.
8. When lifting the pipette tip out of the glass feeding chamber, limit any contact with the cut 15mL conical tube.
9. After 30min remove the ISM from the flask, again limiting any surface contact.
10. Gently use the aspirator gun to suction mosquitoes out of the glass feeding chamber, again limiting any surface contact.
11. Surface decontaminate the outside of the aspirator tip, place it into the protective sheath for transport to the CO_2 knockout chamber within the CL3.

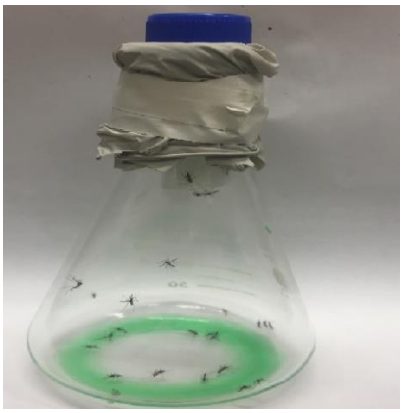


Figure 1- Glass feeding chamber used during sugar infections. A 125 mL Erlenmeyer flask was modified by taping latex gloves over the top and inserting a cut 15mL conical tube. The latex gloves prevent mosquitoes from escaping and the conical tube prevents any surface contamination with a 10mL pipette.

Appendix 4- Detection Protocol testing in mosquito samples

Viral testing was done following the Public Health Agency of Canada's gold standard guidelines and followed the protocols established by Lanciotti et al. (2000) with some minor changes. The BA-1 media was changed to supplemented DMEM (Table 1). Experimental evidence showed no significant difference between the two media types, in regards to loss of viral titer from repetitive freeze/thaws (Giordano, 2014, personal communication). An additional benefit of using this DMEM recipe is that these reagents are also used for cell culture and plaque assay procedures when growing West Nile virus.

The kit used for testing was also changed to iScript One-Step (Bio-Rad Cat. #: 170-8895) as opposed to the QIAamp viral RNA kit (QIAGEN, Valencia, Calif.). In addition to changing kits the suggested 50µL (5µL RNA and 45µL master mix) final volume used during RT-qPCR was also reduced to a final volume of 25µL (5µL RNA and 20µL master mix) per reaction. Preliminary lab experiments showed that substituting the kits and reducing the final volume of the PCR reaction mixture had no effect on the detection of a positive control in a series of dilutions (Larissa Barelli, 2014, personal communication).

4.1 Mosquito Homogenization- RNeasy 96 Kit (Qiagen, Cat. # 74182)

1. Mosquitoes that need to be processed are stored in a -80°C freezer.
2. ALL MOSQUITO WORK MUST BE CONDUCTED IN THE BIOSAFETY CABINET.
3. Add 1mL freshly made sDMEM and 1 copper BB (Copperhead .177-Caliber BBs, Cat. #0737) into a 2mL microfuge tube and close the lid.
4. Add a negative control (new 2mL microfuge tube, 1mL sDMEM, and 1 copper BB) to spot H11

5. Leave slot H12 empty for the RNA extraction positive control.
6. Once finished with all the sDMEM additions, the tubes are then placed into the holders used for the Mixer Mill 300.
7. Turn on the Mixer Mill 300 (switch on back) and homogenize samples for 2 minutes at 30shake/second.
8. Place the Mixer Mill holders (without the adaptors) that contain the tubes that you just homogenized into the 96-well plate centrifuge. Centrifuge @ 6000rpm for 4 minutes.
9. While centrifuging you can prepare a 96-well holding block (1.8mL profile) with 250uL of RLT lysis buffer. Label the plate with the mosquito box number and fill out an extraction card to denote which samples are in which location.
10. Open vials carefully and take out 100uL of mosquito supernatant and place it in the holding block that contains the 250uL RLT lysis buffer prepared previously. Remember to use a new pipette tip every transfer. THIS IS A CRITICAL STEP IN THE PROCEDURE. DON'T MIX UP SAMPLES OR CONTAMINATE ADJACENT SAMPLES WITH THEIR NEIGHBORS.
11. Once all of the mosquito supernatants are transferred to the proper slots in the holding block, cover the block with airpore tape. The left over mosquito homogenates must be placed back into the sample box and into the right slots that they came in. THE BOXES MUST BE PLACED BACK INTO THE -80°C FREEZER IMMEDIATELY. RNA is very sensitive and can degrade very easily.
12. RNA in lysis buffer is fairly stable; it can be stored in the fridge if you cannot directly proceed to the RNA extraction step for several days (4 maximum or at -80°C for several months).

Table 1: Ingredients for supplemented DMEM (sDMEM) for Mosquito Homogenization.

Reagent	Volume Required	[Final]	Catalogue # (company)
DMEM	88 mL	88%	F1015-500ML (Sigma-Aldrich)
(FBS)	10 mL	10%	D6546 (Sigma-Aldrich)
Pen/Strep/Glut (10,000U)	2 mL	2%	103 780 16 (Invitrogen)
Total Volume	***100 mL***		

*Note: Make fresh - shelf life 5 days and light sensitive; store in fridge (4-8°C).

4.2 RNA Extraction- RNeasy 96 Kit (Qiagen, Cat. No. 74182)

1. Thaw one tube of WNV extraction control (contains 100uL of infected cell supernatant in appropriate lysis buffer) for every extraction plate.
2. Add a positive control to well H12 of each 96-well holding block with previously lysed samples.
3. Place a 96-well Filter Plate on top of a 96-well (2 mL, "tall" profile) square-well block. Mark the plate with the box number.
4. Remove the Airpore tape from the 96-well holding block that contains your 100uL sample and 250uL RLT lysis buffer. Add 350uL of 75% EtOH to each well and mix by pipetting up and down 3x while using the 8 sample multichannel pipette.
5. On the last up/down sequence of mixing transfer the entire 700uL of solution to the corresponding row in the RNeasy^{TM} 96-well filter plate on top of a square-well block. The position of each sample must be the same from which it came from (the holding blocks). THIS IS A CRITICAL STEP IN THE PROCEDURE. DON'T MIX UP SAMPLES OR CONTAMINATE ADJACENT SAMPLES WITH THEIR NEIGHBOURS.
6. Change pipette tips every time and repeat steps 4-5 until all samples are transferred.
7. Dispose of the empty holding blocks in a biohazard bag.
8. Place 700uL of RLT + EtOH in spot H11 for the negative control *If more than one box is being processed, only one plate needs to have controls on it.*
9. Seal the RNeasy^{TM} 96-filter plate on top of a tall 96-well block with Airpore tape.
10. Centrifuge at 6000rpm for 4 minutes.
11. Carefully remove the tape and dispose of it and add 800uL of RW1 to each well.
12. Centrifuge 6000rpm for 4 minutes.
13. Remove the RNeasy^{TM} 96-filter plate and place it on top of a new tall 96-well block.
14. Discard the flow-thru down the sink with plenty of water. The square-well block can be reused.

15. Next add 800uL of RPE to each well and seal the plate with Airpore tape.
16. Centrifuge at 6000rpm for 4 minutes.
17. Carefully remove the tape and add another 800uL of RPE. Seal plate with a new Airpore tape.
18. This time centrifuge for 10 minutes at 6000rpm
19. After the 10 minutes remove the RNeasy^{TM} 96-filter plate, dispose of the flow-thru. Place the RNeasy^{TM} 96-filter plate on top of a new tall 96-well block and centrifuge again at 6000rpm for 1 minute. This ensures that the entire wash buffer is removed.
20. Next remove the RNeasy^{TM} 96-filter plate and place it on top of the blue elution rack. Make sure to label the elution rack prior to centrifuging.
21. Remove the Airpore tape.
22. Add 100uL of RNase-free water to each well and seal the plate with new Airpore tape.
23. Let stand for 4 minutes then centrifuge at 6000rpm for 4 minutes
24. Remove the RNeasy^{TM} 96-filter plate and cap the elution rack with the strip caps.
25. Each well now contains total RNA!
26. Use the RNA immediately for RT-qPCR.
27. Store RNA at -80°C when finished. Make sure that the elution racks are labelled accordingly to correspond to their mosquito homogenization box. Also label and place a numbered RNA Extraction card with every elution rack so wells can be identified during future use.

4.3 WNV RT-qPCR Plate set up (Bio-Rad iScript One-Step, Cat. #: 170-8895)

1. Begin thawing reagents and turn on the computer and PCR Machine to give them sufficient time to warm up before running samples.
2. Once the primers and probes are completely thawed create the Master Mix (MM) by adding the reagents to a 1.5 mL microfuge tube (always pipet reagents in order of volume, largest to smallest). Prepare enough MM for the number of mosquito

samples, the negative (3) and positive (3) controls, as well as 5-10% extra for pipet-error.

3. Mix the MM tube thoroughly ensuring that all reagents are evenly distributed.
4. Pipet 15 µL of WNV MM into each required well of the 96-well PCR plate.
5. Add 5 µL of extracted RNA (from blue extraction plate) to the corresponding well on the PCR plate. Change pipette tips every time. **THIS IS A CRITICAL STEP IN THE PROCEDURE. DON'T MIX UP SAMPLES OR CONTAMINATE ADJACENT SAMPLES WITH THEIR NEIGHBORS.**
6. Ensure 5 µL is taken from the extraction controls in well H11 and H12.
7. Add 5 µL of water into 3 negative control wells.
8. Add 5 uL of positive control from three prepared 3 dilutions.
9. Seal the reaction plate with Optical tape. Wipe with a KimWipe to remove any smudges or finger prints that would interfere in the ability of the camera to read the fluorescence.
10. Ensure that there are no air bubbles present. Air bubbles will interfere with the reaction. Centrifuge the plate @ 6,000rpm for 1 minute if necessary (ensure PCR plate is in a holder so the plate isn't damaged).
11. Place the reaction plate into the PCR machine, wipe with a KimWipe again.

Table 2: Bio-Rad iScript One-Step (Cat. #: 170-8895) reagent list.

Reagent	Volume of Stock	[Final]	Example Prep for 100 reactions
2X Buffer	10.0 uL	1X	10.0uL × 100 = 1000 uL
RNase-free water	4.1 uL	-	4.1uL × 100 = 410 uL
P/P solution (Pre Mixed)	0.52 uL		0.52uL × 100 = 52 uL
F-primer (100uM)	(0.5 uL)	1 uM	
R-primer (100uM)	(0.5 uL)	1 uM	
Probe (20uM)	(0.3 uL)	120 nM	
iScript	0.4 uL	-	0.4uL × 100 = 40 uL
			Mix and aliquot 15 uL / well
RNA	5.0 uL		5.0uL
Total volume	***20uL***		***20uL***

**The positive controls: WNv NY99 from extraction elution well H12, and WNv E101 at three dilutions (10^{-1} – 10^{-3}) **

The negative controls: Extraction elution from well H11, RNase free water (3 wells)

Table 3: Primer and Probes used in Bio-Rad iScript One-Step (Cat. #: 170-8895).

Gene	Primer	Sequence (5'-3')	[Stock]	[Final]	Info
3' UTR	Gen-F	CAG ACC ACG CTA CGG CG	100 µM	1 µM	**WNv screening** (10,668-10,684)
	Gen-R	CTA GGG CCG CGT GGG	100 µM	1 µM	(10,770-10,756)
	Gen-probe	FAM-TCTGCGGAGAGTGCAGTCTGCGAT-BHQ	20 µM	120 nM	(10,691-10,714); 102 bp
Envelope (E)	Env-F	TCA GCG ATC TCT CCA CCA AAG	100 µM	1 µM	**WNv confirmation** (1160-1180)
	Env-R	GGG TCA GCA CGT TTG TCA TTG	100 µM	1 µM	(1209-1229)
	Env-probe	FAM-TGCCCGACCATGGGAGAAGCTC-BHQ	20 µM	120 nM	(1186-1207); 101 bp

4.4 WNV RT-qPCR Computer set up

The machines available for real-time is a MyIQ™ Single-Channel, and an iCycler™ Multi-Channel Detection System from BioRad.

1. Camera of the real-time machine must warm for 30 minutes prior to each reaction.
2. Click on the MyIQ or iCycler icon on the computer desktop
3. In the Library Module select the View Protocol tab and click on the thermal protocol file named: WNV#1.tmo (runs for 40 cycles)
4. Select the View Plate Setup and click on a plate setup file. Select the Edit this plate setup and edit the plate according to the samples to be examined. Fill in the unknown samples and positive and negative controls using the appropriate action buttons.
5. Under the fluorophores box select FAM-490.
6. Select the Save this plate setup button and Run with selected protocol button.
7. In the Run prep window, ensure you have selected the correct protocol and plate setup. Also make sure that the sample volume reads 20uL and select collect well factors form experimental plate.
8. Click on Begin Run button to start experiment. A save dialog box will appear, enter an appropriate title for the testing plate, include date of test.
9. The experiment takes approximately 3.0 hours to run. When the run is over an amplification plot and Threshold Cycle (Ct) value will be generated. Click on the Reports button. Under the Select Report (drop down list) select PCR baseline. A

graph and a list of Ct values should appear. If negative controls have Ct values the run is invalid and must be done again.

Ensure that any well with a listed Ct value actually has a correctly shaped S-curve. This means more than just a number! Ct values above 37 is typically where "noise" starts to occur on the graph. PCR protocols with >35 cycles will almost always start to form artifacts (eg. Primer-dimers).

10. Next, click on the **Save to file** button and save the report titled exactly the same as the output file (just change the extension to read .doc instead of .odm).

Table 4: WNV testing protocol for RT-qPCR on the MyIQTM Single-Channel and an iCycler™ Multi-Channel Detection System from BioRad. Protocol is directly taken from the "gold" standard mosquito viral testing protocol established by Lanciotti et al. (2000).

WNv RT-qPCR Conditions		
Protocol Step	Temp	Duration
1. cDNA synthesis	50°C	30 min
2. Denature RT, Activate Taq	95°C	10 min
3.1 Amplification *3.2 Real-time detection* *3.3 Repeat 3.1 and 3.2 (40x)*	90°C 60°C	15 sec 1 min
4. Hold	4°C	Hold

Appendix 5- Cell Culture Protocols

Protocols involving cell culture were modified from original protocols provided by the Public Health Agency of Canada and those found in Podlech et al. (2002).

5.1 Virus Propagation

1. Only begin once an 80-90% confluent monolayer of VERO cells has developed.
2. Discard growth media and wash monolayer with Dulbecco's PBS or with media without serum.
3. Infect the cells with a Multiplicity of Infection (MOI) of 0.02. MOI=(Plaque Forming Units (PFU))/(Number of Cells).
4. Dispense calculated volume of virus stock into 2mL microfuge tube.
5. Bring the volume up to 2 mL with DMEM supplemented with 2% FBS.
6. Incubate infected cells at 37°C, 5% CO2 for one hour
    a. Manually rock the flask or well plates carefully every 15 min
7. Remove infected media and wash with 2mL of DPBS.
8. Remove DPBS and add appropriate amount of DMEM, 2% FBS (v/v) and antibiotics.
    a. 15 mL for T75 flask.
    b. 40 mL for T150 flask.
9. Once significant cytopathic effect is observed in culture (3 days typically), freeze and thaw the flask and its contents at -80°C for 30 min and at room temperature for 20 min.
10. Centrifuge at 800 g for 20 min at 4°C. Parafilm and surface decontaminate any tubes prior to centrifugation. OPTIONAL: More than one flask containing an infected monolayer of VERO cells can be infected to increase the viral titer of working stock.
11. Add FBS to the supernatant to a final concentration of 20%.
12. Aliquot the virus suspension into sterile vials in 0.25, 0.5 or 1.00 mL volumes.
13. Label all vials with: Name of virus, passage number, and date of infection
14. Store at -80°C.

5.2 Plating 6-Well Plates

1. Start with a T-75 flask that is approx. 95-100% confluent.
2. Remove media and rinse the flasks with 5-10 mL of sterile DPBS.
3. Gently rock the flask to distribute the DPBS over the monolayer.
4. Add 3 mL of 0.25% Trypsin-EDTA solution to the monolayer.
5. Rotate the flask so that the trypsin covers the monolayer.
6. Incubate the flask at 37°C, 5% CO2 for 2-4 min.
7. After incubation, vigorously shake the flask from side to side.
8. Check to see if the monolayer has been removed from the surface of the flask. You should be able to see the cells slipping off; the bottom of the flask should no longer be opaque.
9. Immediately add 10 mL of VERO E6 growth media to the flask pipetting up and down vigorously at least 10 times.
10. Be careful not to create excessive air bubbles.
11. Dilute the flask to 42 mL with growth media.
12. Mix frequently by pipetting and add 1.5 mL to each well for a 6 well plate.
13. 0.5 mL for a 12 well plate.
14. The plates should be confluent in approx. 72 hours.

5.3 Virus Dilution

1. Remove vial of frozen WNV stock from the -80°C and allow it to thaw at room temperature.
2. Prepare 5 tubes of 0.9 mL of DMEM supplemented with 2% FBS.
3. Add 100 µL of virus stock solution to the first tube.
4. Pipette to mix and gently invert 1-2 times. Spin down tubes after inversion to ensure that there is no residue in the lid.
5. Repeat the dilution process through all of the 5 tubes.
6. Make sure to change tips between viral dilutions.
7. The effective dilutions of virus now range from 10^{-1} to $10^{-5.}$

5.4 Monolayer Infection

1. Discard growth media from each well and dispose into liquid waste.
2. Rinse cells with 2 mL of DPBS. Gently rock plates and remove DBPS.
3. Add 400 µL of each viral dilution in separate wells.
4. Gently add the virus down the side of each well. It is best to set up each plate as follows:
    a. Working from left to right on the top row: Control, -5 dilution, -4.
    b. Working from left to right on the bottom row: -3, -2, -1.
5. Incubate infected monolayers at 37°C, 5% CO2 for 60 min.
6. Gently rock the plates back and forth several times (approx. every 15 min) during the adsorption period to spread the virus suspension evenly over the cell monolayer and to prevent drying of the cells.
7. During the last 20 min of the incubation period prepare a solution containing a 1:1 ratio of CMC and DMEM supplemented with 2% FBS. Warm the CMC to 37°C prior to mixing with DMEM aids in homogenization.
8. After the incubation period, remove the virus working from low viral titer to high.
9. Wash the wells with 2 mL of DPBS and then remove it.
10. Add 2 mL of CMC/DMEM, 2% FBS solution to each well working from low viral titer to high. Incubate at 37°C, 5% CO2 for desired length.

5.5 CMC Solution Preparation

Prepare in 1L milliQ water as follows:

1. Weigh 32 grams of CMC powder.
2. Fill a 2L beaker with at least 500 mL of milliQ water before adding CMC (or else it will adhere to the bottom of the beaker). Bring the volume up to 1L with milliQ water.
3. Mix with a magnetic stir bar at 150°C for 4 -6 hours, or until fully homogenized, ensuring that it does not boil.
4. Autoclave at 121°C for 10 min.
5. Store at room temperature.

5.6 Crystal Violet Solution

Final concentration of 1% crystal violet (w/v), 20% formaldehyde (v/v), and 30% ethanol (v/v) in 1L of DPBS prepared as follows:

1. Dissolve 10 g of Crystal Violet Powder in 300 mL of 100% ethanol first. Gently mix by rotation and gentle rocking to ensure the powder is fully dissolved.
2. Add 200 mL of Formaldehyde and bring the total volume up to 1L with filter sterilized DPBS.
3. Store in an amber glass bottle and avoid direct contact with light.

5.7 Plaque Assay Staining and Plaque Counting

1. After a 72 hr incubation period the CMC is removed and the cells are stained with a crystal violet solution. 2 mL of the staining solution are used per well.
2. Remove the CMC/DMEM overlay.
3. Wash the wells with 2 mL of DPBS and then remove it.
4. Add 2 mL of crystal violet solution to each well.
5. Incubate in the BSC for 30 min.
6. Wash plates out with water in the sink.
7. Count plaques.

PFU/mL Calculation

Counting plaques allows you to calculate the concentration of the initial viral suspension in PFU per mL (of viral dilution)= (Number of plaques)/(Dilution Factor x (mL of inoculum per plate))

Take the average of all your dilutions for the final PFU/mL calculation. PFU/mL is only an estimate.

Example Calculation:

If 100 µL of the 10^{-8} dilution produced 88 plaques, the titer from that well is:

PFU per mL (of viral dilution)= (88 PFU)/($10^{(-8)}$ x (0.1 mL))

PFU per mL (of viral dilution)= 8.8 x 10^{10} PFU/mL

Appendix 6- Propagation to Detect Virus

1. Ensure that all 6 wells contain an 80-90% confluent VERO E6 monolayer.
2. 5 of the wells will be inoculated with homogenate and 1 well on every plate will contain a negative control.
3. Discard growth media from wells and wash monolayer with Dulbecco's PBS.
4. Infect well with 0.250mL of dissection homogenate or 0.250mL of DMEM for control
5. Incubate cells at 37°C, 5% CO_2 for one hour
    a. Manually rock well plates carefully every 15 min
6. Ensure that no liquid is splashed or dripped into an adjacent well during the subsequent steps.
7. Remove infected media and wash with 2mL of DPBS.
8. Remove DPBS and wash a second time with 2mL of DPBS.
9. Remove DPBS and add 1.5mL supplemented DMEM (Appendix 4, table 1)
10. Incubate cells at 37°C, 5% CO_2 until cytopathic effect is observed.
11. Wells showing signs of cytopathic effect prior to the control well were pipetted into 2mL microfuge tubes, labeled, and immediately stored at -80°C.
12. Well plates were incubated for a maximum of 4 days (Mon-Fri) or until cytopathic effect was observed on the monolayer of control wells.

Appendix 7- Capillary Assay

1. Working inside the plexiglass containment chamber anaesthetize all mosquitoes on the CO_2 plate.
2. One at a time, remove a female from the housing container and place her on the CO_2 plate.
3. Remove the wings and legs (rendering her immobile) and place them into a sterile microfuge tube with 1 mL DMEM.
4. Under the microscope, insert the proboscis into a sterile 5 µL capillary tube filled with a 1:1 mixture of 40% sucrose and FBS.
5. Dissolve 10mg of pilocarpine (VWR Cat. D8537) into 998 µL of PBS and 2 µL of Tween 20 buffer for a 1% solution and apply to thorax.
6. Incubate 10 – 15min.
7. Empty capillary tube into a 1.5mL microfuge tube filled with 0.5mL DMEM and store tube at -80°C. Dispose of capillary tube and needle into sharps container.
8. Place the mosquito into the microfuge tube containing the legs and wings then store tube at -80°C.

Appendix 8- ISM trial results and statistical tests

Table 1- Infection rates from the ISM and infected blood trials. The avian blood feeding data was retrieved from Turell et al. (2001) and the membrane blood feeding data was retrieved from Akhter et al. (1982).

	Infected			
	Rep 1	**Rep 2**	**Rep 3**	**Trial Total**
ISM $10^{5.8}$pfu/mL Females	16/21(76.19%)	18/21 (85.71%)	14/16 (87.50%)	48/58 (82.76%)
ISM $10^{5.8}$ pfu/mL Males	14/17(82.35%)	13/16 (81.25%)	15/18 (83.33%)	42/51 (82.35%)
ISM $10^{3.8}$ pfu/mL Females	10/21(47.62%)	13/25 (52.00%)	13/19 (68.42%)	36/65 (55.38%)
ISM $10^{3.8}$ pfu/mL Males	11/24(45.83%)	4/17 (23.53%)	7/21 (33.33%)	22/62 (35.48%)
$10^{5.8}$ pfu/mL Infected blood meal	4/15 (26.67%)	7/15 (46.67%)	7/18 (38.89%)	18/48 (37.5%)
Avian Host $10^{7.2}$pfu/mL				55/61 (90.16%)
Membrane $10^{4.3}$ pfu/mL				5/19 (26.32%)

Table 2- Infection rates from the ISM and infected blood trials. The avian blood feeding data was retrieved from Turell et al. (2001) and the membrane blood feeding data was retrieved from Akhter et al. (1982).

	Disseminated			
	Rep1	**Rep 2**	**Rep 3**	**Trial total**
ISM $10^{5.8}$pfu/mL Females	16/16 (100%)	18/18 (100%)	14/14 (100%)	48/48 (100%)
ISM $10^{5.8}$ pfu/mL Males	14/14 (100%)	13/13 (100%)	14/15 (93.33%)	41/42 (97.62%)
ISM $10^{3.8}$ pfu/mL Females	9/10 (90.00%)	12/13 (92.31%)	11/13 (84.62%)	32/36 (88.89%)
ISM $10^{3.8}$ pfu/mL Males	10/11(90.91%)	3/4 (75.00%)	6/7 (85.71%)	19/22 (86.36%)
$10^{5.8}$ pfu/mL Infected blood meal	1/4 (25.00%)	3/7 (42.86%)	3/7 (42.86%)	7/18 (38.89%)
Avian Host $10^{7.2}$pfu/mL				47/55 (85.45%)

Table 3- ISM trials Chi-squared p-values. All values below 0.05 are considered significantly different from each other. Data from Table 1 and 2 were used for calculations.

	Infection	Dissemination
ISM $10^{5.8}$pfu/mL Females	0.602	0.427
ISM $10^{5.8}$pfu/mL males	0.987	0.252
ISM $10^{3.8}$pfu/mL Females	0.380	0.356
ISM $10^{3.8}$pfu/mL males	0.100	0.168
Infected blood meal $10^{5.8}$pfu/mL	0.521	0.834

Table 4- ISM infection rate Fisher exact test p-values. All values below 0.05 are considered significantly different from each other. Trial totals data from Table 1 were used for calculations.

ISM $10^{5.8}$pfu/mL Females	1.000	0.002	0	0
	ISM $10^{5.8}$ pfu/mL Males	0.003	0	0
		ISM $10^{3.8}$ pfu/mL Females	0.032	0.086
			ISM $10^{3.8}$ pfu/mL Males	0.844
				$10^{5.8}$ pfu/mL Infected blood meal

Table 5 ISM dissemination rate Fisher exact test p-values. All values below 0.05 are considered significantly different from each other. Trail Totals data from Table 2 were used for calculations.

ISM $10^{5.8}$pfu/mL Females	0.467	0.031	0.028	0
	ISM $10^{5.8}$ pfu/mL Males	0.175	0.113	0
		ISM $10^{3.8}$ pfu/mL Females	1	0.002
			ISM $10^{3.8}$ pfu/mL Males	0.003
				$10^{5.8}$ pfu/mL Infected blood meal

Appendix 10- Laboratory Supplies List

Chemicals

1. 1X Dulbecco's Modified Eagle Medium (DMEM) high glucose, cat.# D6546, Sigma
2. 100X L-glutamine, cat.# 25030-081, Invitrogen (GIBCO)
3. 100X Penicillin/Streptomycin, cat.# 15140-122, Invitrogen (GIBCO)
5. Carboxymethylcellulose sodium salt (CMC), cat.# C4888-500G, Sigma
4. CO2, cat.# CD50, Praxair
5. Crystal Violet, cat.# C0775-25G, Sigma
6. Dulbecco's Phosphate Buffered Saline (DPBS), cat.# D8537, Sigma
7. (+) Pilocarpine hydrochloride solution, cat.# 89152-144, VWR
8. Phosphate Buffered Saline (PBS), cat.# P-8709, Sigma
9. Sucrose, cat.# S0389-1KG, Sigma
10. Virkon Disinfectant/ Cleaner P.W.S Virucide 5 kg, cat.# , 0-2353000, Vetoquinol

Biologicals

1. Citrated Sheep Blood, cat.# CL2581-100D, Cedarlane Labs
2. Fetal Bovine Serum, cat.# F1051, Sigma.
3. JumpStart Taq DNA Polymerase, cat.# D9307, Sigma
4. Reference virus: WNV-Egypt 101, NY99 or equivalent Canadian isolate
5. Hog casings, Loblaws or other distributors
6. VERO E6 cell line, C1008, Kidney, African green monkey, ATCC CRL-1586

Miscellaneous

1. 6 well plate, flat bottom, 17.2 mL, cat. # 83.1839, Sarstedt
2. Capillary Tubes 5 µL, cat.# , Brock Science Stores
3. T-75 flask, canted neck, vented plug cap, cat.# 83.1813.002, Sarstedt
4. Petri dish, 100x15 mm, fully stackable, cat.# 89038-968, VWR

List of References

Adams, B., & Boots, M. (2010). How important is vertical transmission in mosquitoes for the persistence of dengue? Insights from a mathematical model. Epidemics, 2(1), 1-10.

Aitken, T. H., Tesh, R. B., Beaty, B. J., & Rosen, L. (1979). Transovarial transmission of yellow fever virus by mosquitoes (*Aedes aegypti*). The American Journal of Tropical Medicine and Hygiene, 28(1), 119-121.

Akhter, R., Hayes, C. G., Baqar, S., & Reisen, W. K. (1982). West Nile virus in Pakistan. III. Comparative vector capability of *Culex tritaeniorhynchus* and eight other species of mosquitoes. Transactions of the Royal Society of Tropical Medicine and Hygiene, 76(4), 449-453.

Alto, B. W., Reiskind, M. H., & Lounibos, L. P. (2008). Size alters susceptibility of vectors to dengue virus infection and dissemination. The American Journal of Tropical Medicine and Hygiene, 79(5), 688-695.

Anderson, J. F., & Main, A. J. (2006). Importance of vertical and horizontal transmission of West Nile virus by *Culex pipiens* in the northeastern United States. Journal of Infectious Diseases, 194(11), 1577-1579.

Anderson, J. F., Main, A. J., Delroux, K., & Fikrig, E. (2008). Extrinsic incubation periods for horizontal and vertical transmission of West Nile virus by *Culex pipiens pipiens* (Diptera: Culicidae). Journal of Medical Entomology, 45(3), 445-451.

Anderson, J. F., Andreadis, T. G., Main, A. J., Ferrandino, F. J., & Vossbrinck, C. R. (2006). West Nile virus from female and male mosquitoes (Diptera: Culicidae) in subterranean, ground, and canopy habitats in Connecticut. Journal of Medical Entomology, 43(5), 1010-1019.

Anderson, S. L., Richards, S. L., Tabachnick, W. J., & Smartt, C. T. (2010). Effects of West Nile virus dose and extrinsic incubation temperature on temporal progression of vector competence in *Culex pipiens quinquefasciatus*. Journal of the American

Mosquito Control Association, 26(1), 103.

Andreadis, T. G., Anderson, J. F., & Vossbrinck, C. R. (2001). Mosquito surveillance for West Nile virus in Connecticut, 2000: isolation from *Culex pipiens*, *Cx. restuans*, *Cx. salinarius*, and *Culiseta melanura*. Emerging Infectious Diseases, 7(4), 670.

Andreadis, T. G., & Wolfe, R. J. (2010). Evidence for reduction of native mosquitoes with increased expansion of invasive *Ochlerotatus japonicas* (Diptera: Culicidae) in the northeastern United States. Journal of Medical Entomology, 47(1), 43-52.

Angel, B., & Joshi, V. (2008). Distribution and seasonality of vertically transmitted dengue viruses in *Aedes* mosquitoes in arid and semi-arid areas of Rajasthan, India. Journal of Vector Borne Diseases, 45(1), 56.

Aragão, H. D. B. (1929). On infection of the male"*Aedes aegypti*" and the possibility of propagation of yellow fever from *Stegomyia* to *Stegomyia* without passage through man. Memórias do Instituto Oswaldo Cruz, 22, 190-195.

Ardia, D. R., Gantz, J. E., Brent, C., & Strebel, S. (2012). Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. Functional Ecology, 26(3), 732-739.

Baqar, S., Hayes, C. G., Murphy, J. R., & Watts, D. M. (1993). Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. *The American Journal of Tropical Medicine and Hygiene*, *48*(6), 757-762.

Bartlett-Healy, K., Unlu, I., Obenauer, P., Hughes, T., Healy, S., Crepeau, T., ... & Gaugler, R. (2012). Larval mosquito habitat utilization and community dynamics of *Aedes albopictus* and *Aedes japonicus* (Diptera: Culicidae). Journal of Medical Entomology, 49(4), 813-824.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., ... & Myers, M. F. (2013). The global distribution and burden of Dengue. Nature, 496(7446), 504-507.

Blair, C. D. (2011). Mosquito RNAi is the major innate immune pathway controlling

arbovirus infection and transmission. Future Microbiology, 6(3), 265-277.

Boyer, S., Gilles, J., Merancienne, D., Lemperiere, G., & Fontenille, D. (2011). Sexual performance of male mosquito *Aedes albopictus*. Medical and Veterinary Entomology, 25(4), 454-459.

Brady, O. J., Gething, P. W., Bhatt, S., Messina, J. P., Brownstein, J. S., Hoen, A. G., ... & Hay, S. I. (2011). Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Neglected Tropical Diseases, *6*(8), e1760-e1760.

Busvine, J. (2012). Disease transmission by insects: its discovery and 90 years of effort to prevent it. Springer Science & Business Media.

Calisher, C. H. (1994). Medically important arboviruses of the United States and Canada. Clinical Microbiology Reviews, *7*(1), 89-116.

Centres for Disease Control and Prevention. (2016, March 11). The History of Malaria, an Ancient Disease. Retrieved from https://www.cdc.gov/malaria/about/history/.

Chamberlain, R. W., & Sudia, W. D. (1961). Mechanism of transmission of viruses by mosquitoes. Annual Review of Entomology, 6(1), 371-390.

Caminade, C., Medlock, J. M., Ducheyne, E., McIntyre, K. M., Leach, S., Baylis, M., & Morse, A. P. (2012). Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. Journal of the Royal Society Interface, 9(75), 2708.

Choochote, W., Tippawangkosol, P., Jitpakdi, A., Sukontason, K. L., Pitasawat, B., Sukontason, K., & Jariyapan, N. (2001). Polygamy: the possibly significant behavior of *Aedes aegypti* and *Aedes albopictus* in relation to the efficient transmission of dengue virus. The Southeast Asian Journal of Tropical Medicine and Public Health, 32(4), 745-748.

Chow, V. T., Chan, Y. C., Yong, R., Lee, K. M., Lim, L. K., Chung, Y. K., ... & Tan, B. T. (1998). Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes*

albopictus mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. The American Journal of Tropical Medicine and Hygiene, 58(5), 578-586.

Ciota, A. T., & Kramer, L. D. (2013). Vector-virus interactions and transmission dynamics of West Nile virus. Viruses, 5(12), 3021-3047.

Clements, A. N. (1992). The biology of mosquitoes, Volume 1, Development, nutrition and reproduction. Wallingford: CABI Publishing.

Clements, A. N. (1999). The biology of mosquitoes, Volume 2, Sensory reception and behaviour. Wallingford: CABI Publishing.

Clements, A. N. (2011). The biology of mosquitoes, Volume 3, Transmission of viruses and interactions with bacteria. Wallingford: CABI Publishing.

Collins, W. E. (1963). Transmission of St. Louis Encephalitis virus by larval-infected *Culex quinquefasciatus* mosquitoes. Annals of the Entomological Society of America, 56(2), 237-239.

Coz, J., Valade, M., Cornet, M., & Robin, Y. (1976). Transovarian transmission of a Flavivirus, the Koutango virus, in *Aedes aegypti*. Weekly Accounts of the Sessions of the Academy of Sciences. Series D: Natural sciences, 283 (1), 109.

Daep, C. A., Muñoz-Jordán, J. L., & Eugenin, E. A. (2014). Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus. Journal of Neurovirology, 20(6), 539-560.

Davis, C. T., Ebel, G.D., Lanciotti, R.S., Brault, A.C., Guzman, H., Siirin, M., Lambert, A., et al. (2005). Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. Virology, 342(2): 252-265.

De Filette, M., Ulbert, S., Diamond, M. S., & Sanders, N. N. (2012). Recent progress in West Nile virus diagnosis and vaccination. *Veterinary research*, *43*(1), 16.

Desmyter, J., Melnick, J. L., & Rawls, W. E. (1968). Defectiveness of interferon production and of rubella virus interference in a line of African green monkey kidney cells (Vero). Journal of Virology, 2(10), 955-961.

Diamond, M. S. (2009). Progress on the development of therapeutics against West Nile virus. Antiviral Research, 83(3), 214-227.

Dohm, D. J., Sardelis, M. R., & Turell, M. J. (2002). Experimental vertical transmission of West Nile virus by *Culex pipiens* (Diptera: Culicidae). Journal of Medical Entomology, 39(4), 640-644.

Drebot, M. A., Lindsay, R., Barker, I. K., Buck, P. A., Fearon, M., Hunter, F., ... & Artsob, H. (2003). West Nile Virus Surveillance and Diagnostic: A Canadian Perspective. Canadian Journal of Infectious Diseases and Medical Microbiology, 14(2), 105-114.

Finlay, C. (1881). The mosquito hypothetically considered as an agent of yellow fever transmission. Salud publica de Mexico, 34(4), 474-483.

Frith, J. (2012). The history of plague-part 2. The discoveries of the plague bacillus and its vector. Journal of Military and Veterans' Health, 20(3).

Gary, R. E., & Foster, W. A. (2006). Diel timing and frequency of sugar feeding in the mosquito *Anopheles gambiae*, depending on sex, gonotrophic state and resource availability. Medical and Veterinary Entomology, 20(3), 308-316.

Gaunt, M. W., Sall, A. A., de Lamballerie, X., Falconar, A. K., Dzhivanian, T. I., & Gould, E. A. (2001). Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. Journal of General Virology, 82(8), 1867-1876.

Gibson, G., Warren, B., & Russell, I. J. (2010). Humming in tune: sex and species recognition by mosquitoes on the wing. Journal of the Association for Research in Otolaryngology, 11(4), 527-540.

Goddard, L. B., Roth, A. E., Reisen, W. K., & Scott, T. W. (2002). Vector competence of

California mosquitoes for West Nile virus. Emerging Infectious Diseases, 8(12), 1385-1391.

Goddard, L. B., Roth, A. E., Reisen, W. K., & Scott, T. W. (2003). Vertical transmission of West Nile virus by three California *Culex* (Diptera: Culicidae) species. Journal of Medical Entomology, 40(6), 743-746.

Gratz, N. G. (2004). Critical review of the vector status of *Aedes albopictus*. Medical and Veterinary Entomology, 18(3), 215-227.

Gubler, D. J., Kuno, G., Sather, G. E., Velez, M., & Oliver, A. N. D. A. (1984). Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. The American Journal of Tropical Medicine and Hygiene, 33(1), 158-165.

Gubler, D. J. (2007). The continuing spread of West Nile virus in the western hemisphere. Clinical Infectious Diseases, 45(8), 1039-1046.

Gusmão, D. S., Santos, A. V., Marini, D. C., Russo, É. D. S., Peixoto, A. M. D., Bacci Júnior, M., ... & Lemos, F. J. A. (2007). First isolation of microorganisms from the gut diverticulum of Aedes aegypti (Diptera: Culicidae): new perspectives for an insect-bacteria association. *Memórias do Instituto Oswaldo Cruz*, *102*(8), 919-924.

Gwadz, R. W., & Craig, G. B. (1970). Female polygamy due to inadequate semen transfer in *Aedes aegypti*. Mosquito News.

Han, Y. S., Chun, J., Schwartz, A., Nelson, S., & Paskewitz, S. M. (1999). Induction of mosquito hemolymph proteins in response to immune challenge and wounding. Developmental & Comparative Immunology, 23(7), 553-562.

Hardy, J. L., Rosen, L., Kramer, L. D., Presser, S. B., Shroyer, D. A., & Turell, M. J. (1980). Effect of rearing temperature on transovarial transmission of St. Louis encephalitis virus in mosquitoes. The American Journal of Tropical Medicine and Hygiene, 29(5), 963-968.

Hindiyeh, M., Shulman, L. M., Mendelson, E., Weiss, L., Grossman, Z., & Bin, H. (2001). Isolation and characterization of West Nile virus from the blood of viremic

patients during the 2000 outbreak in Israel. Emerging Infectious Diseases, 7(4), 748.

Huhtamo, E., Cook, S., Moureau, G., Uzcátegui, N. Y., Sironen, T., Kuivanen, S., ... & Vapalahti, O. (2014). Novel flaviviruses from mosquitoes: Mosquito-specific evolutionary lineages within the phylogenetic group of mosquito-borne flaviviruses. Virology, 464, 320-329.

Jay, V. (2000). Sir Patrick Manson: father of tropical medicine. Archives of Pathology & Laboratory Medicine, 124(11), 1594-1595.

Jiang, Y., Yan, Z., & Hu, Z. (2006). Experimental study on venereal transmission of Den-1 virus by *Aedes albopictus*. Chinese Journal of Vector Biology and Control, 17(4), 327.

Joshi, V., Mourya, D. T., & Sharma, R. C. (2002). Persistence of dengue-3 virus through transovarial transmission passage in successive generations of *Aedes aegypti* mosquitoes. The American Journal of Tropical Medicine and Hygiene, 67(2), 158-161.

Kasap, H., Alptekin, D., Kasap, M., Güzel, A. I., & Lüleyap, U. (2003). Artificial bloodfeeding of *Anopheles sacharovi* on a membrane apparatus. Journal of the American Mosquito Control Association, 19(4), 367-370.

Kay, B. H., & Carley, J. G. (1980). Transovarial transmission of Murray Valley encephalitis virus by *Aedes aegypti* (L). The Australian Journal of Experimental Biology and Medical Science, 58(5), 501.

Kenney, J., & Brault, A. (2014). The role of environmental, virological and vector interactions in dictating biological transmission of arthropod-borne viruses by mosquitoes. *Advances in virus research*, 89, 39-83.

Knell, R. J., & Webberley, K. M. (2004). Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. Biological Reviews, 79(03), 557-581.

Kobayashi, M., Nihei, N., & Kurihara, T. (2002). Analysis of northern distribution of

Aedes albopictus (Diptera: Culicidae) in Japan by geographical information system. Journal of Medical Entomology, 39(1), 4-11.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., ... & Bunning, M. (2003). Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerging Infectious Diseases, 9(3), 311-322.

Kramer, L. D., Styer, L. M., & Ebel, G. D. (2008). A global perspective on the epidemiology of West Nile virus. Annual Review of Entomology, 53, 61-81.

Kuno, G., Chang, G. J. J., Tsuchiya, K. R., Karabatsos, N., & Cropp, C. B. (1998). Phylogeny of the genus *Flavivirus*. Journal of Virology, 72(1), 73-83.

Lanciotti, R. S., Roehrig, J. T., Deubel, V., Smith, J., Parker, M., Steele, K., ... & Hall, R. A. (1999). Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science*, *286*(5448), 2333-2337.

Lanciotti, R. S., Kerst, A. J., Nasci, R. S., Godsey, M. S., Mitchell, C. J., Savage, H. M., ... & Davis, B. S. (2000). Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. Journal of Clinical Microbiology, 38(11), 4066-4071.

Lothrop, H. D., Wheeler, S. S., Fang, Y., & Reisen, W. K. (2012). Use of scented sugar bait stations to track mosquito-borne arbovirus transmission in California. Journal of Medical Entomology, 49(6), 1466-1472.

Luo, Y. P. (2014). A novel multiple membrane blood-feeding system for investigating and maintaining *Aedes aegypti* and *Aedes albopictus* mosquitoes. Journal of Vector Ecology, 39(2), 271-277.

Mackenzie, J. S., Williams, D. T., & Smith, D. W. (2006). Japanese encephalitis virus: the geographic distribution, incidence, and spread of a virus with a propensity to emerge in new areas. Perspectives in Medical Virology, 16, 201-268.

Mahdy, M. S., Spence, L., Subrahmanyan, T. P., & Joshua, J. M. (1979). Arboviral encephalitides in Ontario with special reference to St. Louis encephalitis, and

overview. Ontario Ministry of Health 343-364.

Martins, V. E. P., Alencar, C. H., Kamimura, M. T., de Carvalho Araujo, F. M., De Simone, S. G., Dutra, R. F., & Guedes, M. I. F. (2012). Occurrence of natural vertical transmission of dengue-2 and dengue-3 viruses in *Aedes aegypti* and *Aedes albopictus* in Fortaleza, Ceará, Brazil. PloS One, 7(7), e41386.

Mavale, M., Parashar, D., Sudeep, A., Gokhale, M., Ghodke, Y., Geevarghese, G., ... & Mishra, A. C. (2010). Venereal transmission of chikungunya virus by *Aedes aegypti* mosquitoes (Diptera: Culicidae). The American Journal of Tropical Medicine and Hygiene, 83(6), 1242-1244.

Miller, B. R., Nasci, R. S., Godsey, M. S., Savage, H. M., Lutwama, J. J., Lanciotti, R. S., & Peters, C. J. (2000). First field evidence for natural vertical transmission of West Nile virus in *Culex univittatus* complex mosquitoes from Rift Valley province, Kenya. The American Journal of Tropical Medicine and Hygiene, 62(2), 240-246.

Mitchell, C. J. (1991). Vector competence of North and South American strains of *Aedes albopictus* for certain arboviruses: a review. Journal of the American Mosquito Control Association, 7(3), 446-451.

Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N. L., Khasnatinov, M., ... & De Lamballerie, X. (2015). New insights into flavivirus evolution, taxonomy and biogeographic history, extended by analysis of canonical and alternative coding sequences. PloS one, 10(2), e0117849.

Mourya, D. T., Gokhale, M. D., Basu, A., Barde, P. V., Sapkal, G. N., Padbidri, V. S., & Gore, M. M. (2001). Horizontal and vertical transmission of dengue virus type 2 in highly and lowly susceptible strains of *Aedes aegypti* mosquitoes. Acta Virologica, 45(2), 67-72.

Mukhopadhyay, S., Kuhn, R. J., & Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. Nature Reviews Microbiology, 3(1), 13-22.

Mulyatno, K. C., Yamanaka, A., Yotopranoto, S., & Konishi, E. (2012). Vertical

transmission of dengue virus in *Aedes aegypti* collected in Surabaya, Indonesia, during 2008–2011. Japanese Journal of Infectious Diseases, 65(3), 274-276.

Nation, J. L. (2008). Insect physiology and biochemistry. CRC press.

Ogden, N. H., Milka, R., Caminade, C., & Gachon, P. (2014). Recent and projected future climatic suitability of North America for the Asian tiger mosquito *Aedes albopictus*. Parasites & Vectors, 7(1), 532.

Paupy, C., Delatte, H., Bagny, L., Corbel, V., & Fontenille, D. (2009). *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes and Infection, 11(14), 1177-1185.

Payne, A. F., Binduga-Gajewska, I., Kauffman, E. B., & Kramer, L. D. (2006). Quantitation of flaviviruses by fluorescent focus assay. Journal of Virological Methods, 134(1), 183-189.

Perera, R., Khaliq, M., & Kuhn, R. J. (2008). Closing the door on flaviviruses: entry as a target for antiviral drug design. Antiviral Research, 80(1), 11-22.

Petersen, L. R., & Roehrig, J. T. (2001). West Nile virus: a reemerging global pathogen. *Emerging Infectious Diseases, 7*(4).

Podlech, J., Holtappels, R., Grzimek, N. K., & Reddehase, M. J. (2002). Animal models: murine cytomegalovirus. Methods in Microbiology, 32, 493-IN11.

Poidinger, M., Hall, R. A., & Mackenzie, J. S. (1996). Molecular characterization of the Japanese encephalitis serocomplex of the flavivirus genus. Virology, 218(2), 417-421.

Public Health Ontario. (2012). Vector Borne Diseases Summary Report 2012. Ontario Agency for Health Protection and Promotion. Retrieved from https://www.publichealthontario.ca/en/BrowseByTopic/InfectiousDiseases/Pages/IDLandingPages/West-Nile-Virus.aspx.

Public Health Ontario, (2013). Vector Borne Diseases Summary report 2013. Ontario

Agency for Health Protection and Promotion. Retrieved from https://www.publichealthontario.ca/en/BrowseByTopic/InfectiousDiseases/Pages/IDLandingPages/West-Nile-Virus.aspx.

Reisen, W. K., Fang, Y., Lothrop, H. D., Martinez, V. M., Wilson, J., O'Connor, P., ... & Brault, A. C. (2006). Overwintering of West Nile virus in southern California. Journal of Medical Entomology, 43(2), 344-355.

Richards, S. L., Ponnusamy, L., Unnasch, T. R., Hassan, H. K., & Apperson, C. S. (2006). Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of central North Carolina. Journal of Medical Entomology, 43(3), 543-551.

Rios, M., Añez, G., Chancey, C., & Grinev, A. (2012). Dengue Virus And Other Arboviruses: A Global View Of The Risks. Vox Sanguinis, 103, 62-63.

Rohani, A., Zamree, I., Lee, H. L., Mustafakamal, I., Norjaiza, M. J., & Kamilan, D. (2007). Detection of transovarial dengue virus from field-caught *Aedes aegypti* and *Ae. albopictus* larvae using C6/36 cell culture and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques. *Dengue Bulletin* 31: 47–57.

Rosen, L., & Gubler, D. (1974). The use of mosquitoes to detect and propagate dengue viruses. American Journal of Tropical Medicine and Hygiene, 23(6), 1153-60.

Rosen, L. (1987). Sexual transmission of dengue viruses by *Aedes albopictus*. The American Journal of Tropical Medicine and Hygiene, *37*(2), 398-402.

Rosen, L. (1988). Further observations on the mechanism of vertical transmission of flaviviruses by *Aedes* mosquitoes. American Journal of Tropical Medicine and Hygiene, *39*(1), 123-126.

Rosen, L., Lien, J. C., Shroyer, D. A., Baker, R. H., & Lu, L. C. (1989). Experimental vertical transmission of Japanese encephalitis virus by *Culex tritaeniorhynchus* and other mosquitoes. The American Journal of Tropical Medicine and Hygiene, 40(5), 548.

Sardelis, M. R., Turell, M. J., Dohm, D. J., & O'Guinn, M. L. (2001). Vector competence of selected North American *Culex* and *Coquillettidia* mosquitoes for West Nile virus. Emerging Infectious Diseases, 7(6), 1018.

Sardelis, M. R., Turell, M. J., O'Guinn, M. L., Andre, R. G., & Roberts, D. R. (2002). Vector competence of three North American strains of *Aedes albopictus* for West Nile virus. Journal of the American Mosquito Control Association, 18(4), 284-289.

Shi, P. Y., Kauffman, E. B., Ren, P., Felton, A., Tai, J. H., Dupuis, A. P., ... & Ebel, G. D. (2001). High-throughput detection of West Nile virus RNA. Journal of Clinical Microbiology, 39(4), 1264-1271.

Shroyer, D. A. (1990). Venereal transmission of St. Louis encephalitis virus by *Culex quinquefasciatus* males (Diptera: Culicidae). Journal of Medical Entomology, 27(3), 334-337.

Sigma Life Sciences. (2008). qPCR Technical Guide. Sigma-Aldrich Co. pp.1–40.

Sota, T., & Mogi, M. (1992). Survival time and resistance to desiccation of diapause and non-diapause eggs of temperate *Aedes* (*Stegomyia*) mosquitoes. Entomologia Experimentalis et Applicata, 63(2), 155-161.

Styer, L. M., Kent, K. A., Albright, R. G., Bennett, C. J., Kramer, L. D., & Bernard, K. A. (2007). Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. PLoS Pathology, 3(9), e132.

Suthar, M. S., Diamond, M. S., & Gale Jr, M. (2013). West Nile virus infection and immunity. Nature Reviews Microbiology, 11(2), 115-128.

Thielman, A., & Hunter, F. F. (2006). Establishment of *Ochlerotatus japonicus* (Diptera: Culicidae) in Ontario, Canada. Journal of Medical Entomology, 43(2), 138-142.

Thielman, A. & Hunter, F.F. (2007). Photographic Key to the Adult Female Mosquitoes (Diptera: Culicidae) of Canada. Canadian Journal of Arthropod Identification, 4(4).

Thompson, W. H., & Beaty, B. J. (1978). Venereal transmission of La Crosse virus from male to female *Aedes triseriatus*. The American Journal of Tropical Medicine and Hygiene, 27(1 Pt 1), 187-196.

Thongrungkiat, S., Wasinpiyamongkol, L., Maneekan, P., Prummongkol, S., & Samung, Y. (2012). Natural transovarial dengue virus infection rate in both sexes of dark and pale forms of *Aedes aegypti* from an urban area of Bangkok, Thailand. Southeast Asian Journal of Tropical Medicine & Public Health, 43(5), 1146-1152.

Tiawsirisup, S., Platt, K. B., Evans, R. B., & Rowley, W. A. (2004). Susceptibility of *Ochlerotatus trivittatus* (Coq.), *Aedes albopictus* (Skuse), and *Culex pipiens* (L.) to West Nile virus infection. Vector-Borne & Zoonotic Diseases, 4(3), 190-197.

Tun-Lin, W., Burkot, T. R., & Kay, B. H. (2000). Effects of temperature and larval diet on development rates and survival of the dengue vector *Aedes aegypti* in north Queensland, Australia. Medical and Veterinary Entomology, *14*(1), 31-37.

Turell, M. J., O'Guinn, M. L., Dohm, D. J., & Jones, J. W. (2001). Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. Journal of Medical Entomology, 38(2), 130-134.

Turell, M. J., Spring, A. R., Miller, M. K., & Cannon, C. E. (2002). Effect of holding conditions on the detection of West Nile viral RNA by reverse transcriptase-polymerase chain reaction from mosquito (Diptera: Culicidae) pools. Journal of Medical Entomology, 39(1), 1-3.

Turell, M. J., Dohm, D. J., Sardelis, M. R., O'guinn, M. L., Andreadis, T. G., & Blow, J. A. (2005). An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology, 42(1), 57-62.

Unlu, I., Mackay, A. J., Roy, A., Yates, M. M., & Foil, L. D. (2010). Evidence of vertical transmission of West Nile virus in field-collected mosquitoes. Journal of Vector Ecology, 35(1), 95-99.

Vaidyanathan, R., Fleisher, A. E., Minnick, S. L., Simmons, K. A., & Scott, T. W. (2008). Nutritional stress affects mosquito survival and vector competence for West Nile virus. Vector-Borne and Zoonotic Diseases, 8(6), 727-732.

Venita Jay (2000) Sir Patrick Manson. Archives of Pathology & Laboratory Medicine: November 2000, Vol. 124, No. 11, pp. 1594-1595.

White, L. A. (1987). Susceptibility of *Aedes albopictus* C6/36 cells to viral infection. Journal of Clinical Microbiology, 25(7), 1221-1224.

WHO. (2016). Vector-Borne Diseases. WHO Fact Sheet. Available at: http://www.who.int/mediacentre/factsheets/fs387/en/.

Wood, D. M., Dang, P. T., & Ellis, R. A. (1979). The insects and arachnids of Canada. Part 6. The mosquitoes of Canada. Diptera: Culicidae. Canadian Government Publishing Centre.